Patent Office
Canberra

I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002952747 for a patent by LUDWIG INSTITUTE FOR CANCER RESEARCH as filed on 18 November 2002.

WITNESS my hand this
Twenty-fifth day of November 2003

JANENE PEISKER
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

Ludwig Institute for Cancer Research

PROVISIONAL SPECIFICATION

Invention Title:

Method for analysing peptides

The invention is described in the following statement:

FIELD OF THE INVENTION

The present invention is concerned with a method for the analysis of proteins and peptides.

BACKGROUND

With completion of the first draft of the human genome sequence, the challenge now facing researchers is to understand gene function. However, the biological function of a gene cannot be determined from a simple examination of its DNA sequence. Comprehensive analysis of the proteins expressed by the genome, therefore, promises to bridge the gap between the gene and its biological function. The term proteomics has become synonymous with (i) the identification and characterization of all proteins synthesized by a particular cell type or tissue at any given time, and quantitation of the global changes in protein expression levels observed between two different cell states (collectively known as expression proteomics) and, (ii) with the identification of components of functionally active protein complexes and characterization of the intricate protein-protein interactions involved in intracellular protein trafficking and signaling pathways (collectively known as cell-mapping proteomics). Taken together, these approaches allow comprehensive examination, at the protein level, of the complex cellular changes that occur following transformation of cells from one state to another [Blackstock, W.P. and Weir, M.P. *Trends Biotechnol.* **1999**, *17*, 121-127.; Pandey, A. and Mann, M. *Nature* **2000**, *405*, 837-846.].

Recent developments in mass spectrometry (MS), coupled with the development of sophisticated bioinformatic tools for database interrogation of MS derived data, have been the major factors enabling proteomics [Mann, M., Hendrickson, R.C. and Pandey, A. *Annu. Rev. Biochem.* **2001**, *70*, 437-473.]. In particular, the speed, specificity, and sensitivity of mass spectrometry make it especially attractive for use in strategies requiring rapid protein identification and characterization. Conventional MS approaches to proteomics generally involve one or two-dimensional electrophoretic (2DE) separation of protein mixtures, after which the protein spot or gel slice is cut out and subjected to *in situ* proteolysis using trypsin. Peptides are then extracted and subjected to mass spectrometric analysis. The masses of these peptides are characteristic of the protein, and provide a peptide "mass fingerprint" which can be used in database searches to identify the protein [Henzel, W.J. Billeci T.M., Stults J.T.,

Wong S.C., Grimley C. and Watanabe C. *Proc. Natl. Acad. Sci. USA.* **1993**, *90*, 5011-5015.]. A more comprehensive approach, particularly for the identification and quantitation of individual components present in complex protein mixtures, is to subject each of the proteolytically derived peptides to tandem mass spectrometry. Subsequent identification of each peptide may be performed by either database analysis of the uninterpreted product ion spectrum [Eng, J.K. McCormack, A.L. and Yates, J.R. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 976-989.]; through database searching of a partially derived amino acid "sequence tag" [Mann, M. and Wilm, M. *Anal. Chem.* **1994**, *66*, 4390-4399.], or by "de-novo" sequence analysis [Hunt, D. F., Yates, J.R., Shabanowitz J., Winston S. and Hauer, C.R. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 6233-6237.].

This general analysis strategy is complicated however, by several limitations.

Foremost is that 2D-gels are only capable of resolving approximately 1500-2000 proteins, yet there are typically greater than 8000 proteins expressed per cell. Therefore, only the most abundant proteins are observed and a significant portion of 2-DE separated gel spots contain more than one protein due to co-electrophoresis and/or differentially modified (or processed) forms of the same protein.Additionally, several classes of proteins, notably hydrophobic proteins, low abundance proteins, and those with extremes of pI and molecular weight are poorly represented in 2D-gel based separations [Gygi, S.P., Corthals, G.L., Zhang, Y., Rochon, Y., Aebersold, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9390-9395.]. Also, 2-DE is labor-intensive, slow, technically demanding, often suffers from poor reproducibility, and hence is not readily amenable to high-throughput or automation.

Secondly, while the identification of only one peptide from a protein digest by MS/MS is required to unambiguously identify that protein, provided that the peptide is unique to a single protein, in practice a significant amount of time during the analysis of complex peptide mixtures is spent in analyzing either the same peptide ion (e.g., in sequential scans during chromatographic separation of a complex peptide mixture) or different peptides of the same protein. Routinely therefore, peptides present at high relative abundance are preferentially sampled, and information regarding the identity of proteins represented in the complex mixture as low abundance peptides is commonly not obtained. Also, up to a quarter of peptide MS/MS spectra obtained during

MS based peptide sequencing are unassignable (i.e., product ion spectra are too complex, too low in abundance, or lack sufficient ions to enable their interpretation) [Simpson, R.J., Connelly, L.M., Eddes, J.S., Pereira, J.J., Moritz, R.L. and Reid, G.E. *Electrophoresis.* **2000**, *21*, 1707-1732].

In order to partially overcome these limitations, dynamic exclusion during MS/MS acquisition has been employed to allow greater numbers of distinct peptide ions to be selected throughout the course of an LC/MS/MS experiment [Davis, M.T.; Spahr, C.S.; McGinley, M.D.; Robinson, J.H.; Bures, E.J.; Beierle, J.; Mort, J.; Yu, W.; Luethy, R.; Patterson, S.D. *Proteomics* **2001**, *1*, 108-117.]. Several strategies for simplification of the peptide mixture prior to mass spectrometric analysis, either by multidimensional chromatographic methods [Washburn, M.P., Wolters, D. and Yates, J.R. *Nat. Biotechnol.* **2001**, *19*, 242-247.], or by selective enrichment in solution of only those peptides containing certain amino acids, by affinity selection [Spahr, C.S. Susin, S.A., Bures, E.J., Robinson, J.H., Davis, M.T., McGinley, M.D., Kroemer, G. and Scott D. Patterson, S.D. *Electrophoresis.* **2000**, *21*, 1635-1650] or by differential chromatography [Gevaert, K., Van Damme, J., Goethals, M., Thomas, G.R., Hoorelbeke, B., Demol, H., Martens, L., Puype, M., Staes, A. and Vandekerckhove, J. *Mol. Cell. Proteomics.* 2002, In press.], have also been described. In combination with the dynamic exclusion and multidimensional chromatographic approached outlined above, these methods allow further increases in the number of proteins that can be identified by mass spectrometric analysis. However, the ability to identify low abundance proteins in these complex mixtures, where the expression of individual proteins may vary from 10 to 10^6 copies per cell, including those potentially containing important information such as the identity and location of post-translational modifications, still remains a significant challenge.

A fundamental aspect of proteomics research is the determination of protein expression levels between two different states of a biological system (i.e., relative quantification of protein levels), such as that encountered between a normal and diseased cell or tissue. As there is a marked disparity between changes in mRNA expression levels (transcriptomics) and their corresponding proteins (proteomics), it is clear that array-based gene expression monitoring or other gene expression methods for measuring mRNA abundances, alone, are insufficient for analyzing the cell's protein complement [Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. *Mol. Cell. Biol.* **1999**, 19, 1720-1730.].

Qualitative and quantitative analysis of changes in protein expression profiles as a function of, for example, cell-cycle regulation, disease state or drug exposure has traditionally been performed by 2DE via image analysis of individual stained protein spots, due to the high dimensionality afforded by the orthogonal isoelectric point and molecular weight separation modes [Patton & Beechem *Curr Opinion Chem Biol* **2002**, *6*, 63-69.; Zhou, G., Li, H., DeCamp, D., Chen, S., Shu, H., Gong, Y., Flaig, M., Gillespie, J.W., Hu, N., Taylor, P.R., Emmert-Buck, M.R., Liotta, L.A., Petricoin, E.F. and Zhao, Y. *Mol Cell Proteomics* **2002** *1*, 117-123]. However, these 2DE gel based quantitation approaches suffer from the same limitations as those discussed above for 2DE gel based protein identification, thereby limiting quantitative analysis to those proteins that are present as relatively abundant, pure spots on the gel and that can be adequately visualized by the staining method employed [Gygi, S.P., Corthals, G.L., Zhang, Y., Rochon, Y., Aebersold, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9390-9395.]. Some of these shortcomings have been overcome by the use of more sensitive detection methods, such as the SYPRO ruby fluorescent protein stain [Berggren, K., Chernokalskaya, E., Steinberg, T.H., Kemper, C., Lopez, M.F., Diwu, Z., Haugland, R.P. and Patton, W.F. *Electrophoresis* **2000**, 21, 2509-2521], and by the use of 2-D difference gel electrophoresis (DIGE) [Unlu, M., Morgamn, M.E. and Minden, J.S. *Electrophoresis*, **1997**, 18, 2071-2077.]. However, these methods are fundamentally reliant on the use of adequate software packages for spot detection, gel matching and spot quantitation [Raman, B., Cheung, A. and Martin, M.R. *Electrophoresis*, **2002**, *23*, 2194-2202.] and cannot account for situations where overlapping protein spots are present.

Two general approaches for MS based quantitation of differential protein expression levels between two different cell/tissue states, using isotopic labeling, have been developed. First, the groups of Smith [Pasa-Tolic, L; Jensen, P. K.; Anderson, G. A.; Lipton, M. S.; Peden, K. K.; Martinovic, S; Tolic, N; Bruce, J. E.; Smith, R. D. *J. Am. Chem. Soc.* **1999**, *121*, 7949-7950.; Veenstra, T.D., Martinovic, S., Anderson, G.A., Pasa-Tolic, L. and Smith, R.D. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 78-82.; Conrads, T.P. *et. al. Anal. Chem.* **2001**, *73*, 2132-2139; Smith, R.D.; Anderson, G.A.; Lipton, M.S.; Pasa-Tolic, L.; Shen, Y.; Conrads, T.P.; Veenstra, T.D.; Udseth, H.R. *Proteomics* **2002**, *2*, 513-523.], Chait [Oda, Y.; Huang, K.; Cross, F. R.; Cowburn, D.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6591-6596.], and others [Chen, X.,

Smith, L.M., Bradbury, E.M. *Anal. Chem.* **2001**, *72*, 1134-1143; Ong, Shao-En., Blagoev, B., Kratchmarova, I., Bach-Kristensen, D., Steen, H., Pandey, A. and Mann, M. *Mol. Cell. Proteomics*. 2002, 1, 376-386; Jiang, H. and English, A.M. J. Proteome. Res. 2002, 1, 345-350; Zhu, H., Hunter, T.C., Pan, S., Yau, P.M., Bradbury, E.M. and Chen, X. Anal. Chem. 2002, 74, 1687-1694.] have all separately presented results involving the metabolic incorporation of isotopically depleted (^{13}C-, ^{15}N-, and ^{2}H-depleted) or enriched amino acids (either by uniform labeling with ^{15}N, or by incorporation of selected amino acids containing heavy isotopes (eg., ^{13}C, ^{15}N, ^{2}H)) into a cellular protein population. After isolation of proteins from the cellular matrix, the sample is combined with one incorporating natural isotopes, then individual proteins are resolved by electrophoretic or chromatographic methods, digested and the masses of the peptides determined by MS. The site specific labels allow efficient identification of those peptides containing the enriched amino acid mass "tags", via comparison with their unlabelled forms. Additionally, by comparing the relative abundances of the peptides from the isotopically enriched sample with those from a sample prepared using naturally abundant isotopes, quantitation of changes in the level of protein expression between the two samples may be obtained. This *in vivo* labelling approach is limited, however, to those systems where cells can be cultured under conditions suitable for incorporation of the isotopic label.

The second approach involves *in vitro* chemical derivatization with isotopically enriched labels following isolation of the proteins from the cellular matrix. One approach that has received much attention to date is the isotope coded affinity tag (ICAT) technique developed by Aebersold and co-workers [Gygi, S. P.; Rist, B; Gerber, S. A.; Turecek, F; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994-999.; Smolka, M; Zhou, H; Aebersold, R. *Mol. Cell. Proteomics*. **2002**, *1*, 19-29.; Shiio, Y; Donohoe, S; Yi, E.C.; Goodlett, D.R.; Aebersold, R; Eisenman, R.N. *EMBO* **2002**, *21*, 5088-5096.; Han, D.K., Eng, J., Zhou, H. and Aebersold, R. *Nature Biotechnol.* **2001**, *19*, 946-951.; Griffin, T.J.; Han, D.K.M., Gygi, S.P., Rist, B., Lee, H., Aebersold, R. and Parker, K.C. *J. Am. Soc. Mass. Spectrom.* **2001**, *12*, 1238-1246.; Gygi, S.P., Rist, B. and Aebersold, R. *Current Opinions in Biotechnol.* **2000**, *11*, 396-401.]. Proteins from two different cell/tissue states are reduced and S-alkylated with either naturally abundant (light) or isotopically enriched (heavy) ICAT reagents, respectively, each containing a biotin moiety for subsequent affinity selection of

cysteine-containing peptides by streptavidin affinity purification, leading to simplification of the mixture prior to MS analysis. In the same manner as described above for metabolic labelled samples, the abundance ratios of the proteolytically derived peptides containing the heavy isotope "diseased" sample compared to those originating from the light isotope "normal" sample (or between those containing structural labels) for each of these methods are indicative of changes in the level of protein expression between the two samples, thereby allowing their differential quantitation.

A number of similar strategies for selective peptide identification and differential quantitation have since been described [Spahr, C.S. Susin, S.A., Bures, E.J., Robinson, J.H., Davis, M.T., McGinley, M.D., Kroemer, G. and Patterson, S.D. *Electrophoresis.* **2000**, *21*, 1635-1650.; Adamczyk, M. and Gebler, J.C.; Wu, J. *Rapid. Commun. Mass Spectrom.* **1999**, *13*, 1813-1817.; Sechi, S. *Anal. Chem.* **1998**, 70, 5750-5158.; Sechi, Salvatore. *Rapid Commun. Mass Spectrom.* **2002**, 16, 1416-1424.; Gehanne, S., Cecconi, D., Carboni, L., Giorgio Righetti, P., Domenici, E. and Handam, M. *Rapid Commun. Mass Spectrom.* **2002**, 16, 1692-1698.; Wang, S.; Regnier, F.E. *J. Chrom. A* 2001, 924(1-2), 345-357.; Amini, A; Chakraborty, A; Regnier, Fred E.. *J. Chrom. B.* 2002, 772, 35-44.; Wang, S., Zhang X. and Regnier, F.E. *J. Chrom. A.* **2002**, 949, 153-162.; Mirgorodskaya, O.A. Kozmin, Y.P., Titov, M.I., Korner, R., Sonksen, C.P. and Roepstorff, P. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1226-1232.; Stewart, I.I.; Thomson, T; Figeys, D. *Rapid Commun. Mass Spectrom.* **2001**, 15, 2456-2465.; Yao, X.; Freas, A; Ramirez, J.; Demirev, P.A.; Fenselau, C. *Anal. Chem.* **2001**, 73, 2836-2842.; Peters, E.C., Horn, E.C., Tully, D.C. and Brock, A. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2387-2392.; Munchbach, M., Quadroni, M., Miotto, G. and James, P. *Anal. Chem.* **2000**, *72*, 4047-4057; Chakraborty, A; Regnier, F. E. *J. Chrom. A* **2002**, 949, 173-184.; Geng, M.; Ji, J.; Regnier, F. E.. *J. Chrom. A* 2000, 870, 295-313.; Ji, J.; Chakraborty, A.; Geng, M.; Zhang, X.; Amini, A.; Bina, M.; Regnier, F. *J. Chrom. B* **2000**, 745, 197-210.; Goodlett, D.R., Keller, A., Watts, J.D., Newitt, R., Yi, E.C., Purvine, S., Eng, J., von Haller, P.; Aebersold, R. and Kolker, E. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1214-1221.; Liu, P; Regnier, F.E. *J. Proteome Res.* **2002**, *1*, 443-450.]. More recently, two groups have described covalent solid phase cysteine capture methods for mixture simplification. [Zhou, H.; Ranish, J. A.; Watts, J. D.; Aebersold, R. *Nature Biotechnol.* **2002**, *20*, 512-515.; Qiu, Y; Sousa, E. A.; Hewick, R. M.; Wang, J.

H. *Anal. Chem.* 2002, 74, 4969-4979.]. Non-isotopic labelling strategies employing structural labelling [Cagney, G., Emili, A. *Nature Biotechnol.* **2002**, *20*, 163-170.; Beardsley, R.L. and Reilly, J.P. *J. Proteome Res.* **2002**, *1*, In Press.], as well as a method for performing differential quantitation by comparing the chromatographically resolved MS ion abundances between a sample of interest and a control have also been described [Bondarenko, P.V., Chelius, D. and Shaler, T.A. *Anal. Chem.* 2002, 74, 4741-4749.; Chelius, D. Bondarenko. P.V. *J. Proteome Res.* 2002, 1, 317 – 323.]. A number of reviews have compared the relative merits and limitations of several of these approaches [Moseley, M.A. *Trends in Biotechnol.* **2001**, 19, S10-S16.; Patton, W.F., Schulenberg, B. and Steinberg, T.H. *Curr. Opin. Biotechnol.* **2002**, 13, 321-328.; Turecek, F. *J. Mass Spectrom.* **2002**, 37, 1-14.; Regnier, F. E.; Riggs, L.; Zhang, R.; Xiong, L.; Liu, P.; Chakraborty, A.; Seeley, E.; Sioma, C.; Thompson, R. A. *J. Mass Spectrom.* 2002, 37, 133-145.].

The two major post translational modifications (PTM's) of proteins are phosphorylation and glycosylation. Of these, the reversible phosphorylation of proteins ranks among the most important PTM that occurs in the cell. Commonly, two approaches for enrichment of phosphoprotein/phosphopeptides prior to their detection and subsequent microcharacterisation have been employed. There are (i) *Immunoprecipitation*; [Gronborg, M., Kristiansen, T.Z., Stensballe, A., Andersen, J.S., Ohara, O., Mann, M., Jensen, O.N. and Pandey, A. *Mol. Cell. Proteomics.* **2002**, In Press.], and (ii) *Immobilized metal ion affinity chromatography (IMAC)*; [Posewitz, M.C. and Tempst, P. *Anal. Chem.* **1999**, *71*, 2883-2892.; Ficarro, Scott B.; McCleland, Mark L.; Stukenberg, P. Todd; Burke, Daniel J.; Ross, Mark M.; Shabanowitz, Jeffrey; Hunt, Donald F.; White, Forest M. *Nat Biotechnol.* **2002**, *20*, 301-305.].

Proteins resolved by 2D gels or 1D SDS-PAGE can be detected by autoradiography or storage phosphorimaging using *in vivo* or *in vitro* ^{32}P labelling [Ji H, Baldwin GS, Burgess AW, Moritz RL, Ward LD, and Simpson RJ. *J Biol Chem* **1993**, *268*, 13396-13405.; Boyle W.J., Geer Van der P. and Hunter T. 1991. *Methods Enzymol.* **201:** 110-149.; Yan J.X., Packer N.H., Gooley A.A. and Williams K.L. 1998. *J. Chromatogr.* **808:** 23-41.], or by western blotting using antibodies to detect phosphorylated proteins. Historically, sequencing of ^{32}P radiolabelled peptides was performed by Edman degradation [Wettenhall, R. E., Aebersold, R. H., and Hood, L. E. *Methods*

Enzymol **1991**, *201*, 186-99.]. However, this approach typically requires prior knowledge of the protein sequence in order to correlate a loss of radioactivity, indicative of the phosphorylation site, with the amino acid sequence of the peptide and can be hampered by a low stoichiometry of phosphorylation at a given site [Katze, M. G., Kwieciszewski, B., Goodlett, D. R., Blakely, C. M., Nedderman, P., Tan, S-L, Aebersold, R. *Virology*. **2000**, *278*, 501-513], or by the presence of multiple differentially phosphorylated forms of the same protein being present [Storm, S. M., and Khawaja, X. Z. *Brain. Res. Mol. Brain. Res.* **1999**, *71*, 50-60.].

More recently, mass spectrometry has proven to be particularly useful for the analysis of protein phosphorylation [Neubauer, G. and Mann, M. *Anal Chem* **1999** 71, 235-242.]. However, the mass spectrometric analysis of phosphopeptides is more complicated compared to that for unmodified peptides, due to their lower ionization efficiencies in positive ion MS analysis mode [Liao P.C., Leykam J., Andrews P.C., Cage D.A. and Allison J. 1994. *Anal. Biochem.* **219:** 9-20.], and the difficulties encountered in rapidly switching between ionisation polarities, to allow both identification (in negative mode) and characterization (in positive mode), during the course of a single experiment [Janek K., Wenschuh H., Bienert M., and Krause E. 2001. *Rapid Commun. Mass Spectrom.* **15**: 1593-1599.; Ma Y., Lu Y., Mo W., and Neubert T.A.. *Rapid Commun. Mass Spectrom.* 2001, **15**, 1693-1700.]. Also, phosphoserine- and phosphothreonine-containing peptides can readily undergo the facile loss of phosphoric acid (H_3PO_4) upon ESI and MALDI ionization, and upon low energy CID.

There are several ways to circumvent the difficulties associated with ionization suppression of phosphopeptides in positive ion mode. The sample may be enriched for phosphopeptides, as discussed above, in order to reduce the excess of unmodified peptides that suppress ionisation. However, regardless of attempts made to overcome signal suppression in the analysis of phosphopeptides, limitations still exist when attempting to identify the site of phosphorylation by tandem mass spectrometry, due to the lability of the phosphate side chain. A simple solution is to perform analysis of the phosphopeptide samples by the parallel analysis of samples previously treated with and without alkaline phosphatase [Larsen, M.R., Sorensen, G.L., Fey, S.J., Larsen, P.M. and Roepstorff, P. *Proteomics*, **2001**, *1*, 223-238.]. More commonly, phosphopeptide identification is performed by precursor ion scan

mode monitoring of the characteristic phosphate specific product ions at m/z 79 (PO_3^-), and m/z 89 ($H_2PO_4^-$), following collision induced dissociation (CID) of the phosphopeptide ions in negative ion mode, or by monitoring for the loss of H_3PO_4 (98 Da) or HPO_3 (80 Da) in positive ion neutral loss scan mode [Carr S.A:, Huddleston M.J., and Annan R.S. 1996. *Anal. Biochem.* **239**: 180-192.; Schlosser A., Pipkorn R., Bossemeyer D., and Lehmann W.D. 2001. *Anal. Chem.* **73**: 170-176.].

Product ion scan mode MS/MS of the intact phosphopeptide can sometimes be used to characterize the precise site of phosphate attachment within the peptide, as the b- and y-type fragment ions formed from peptide backbone cleavage may contain information on the specific location of the phosphorylated amino acid residue. However, as indicated above, this technique is often hampered by extensive gas-phase dephosphorylation and elimination of phosphoric acid, making it difficult to unambiguously locate the site of modification. This is a major problem for the identification of serine- and threonine- phosphorylation, where the dominant loss of phosphoric acid (H_3PO_4, 98 Da) can often constitute the major product ion from the selected precursor. The phosphotyrosine side chain, however, is relatively stable under MS and MS/MS conditions due to the relatively high stability of the arylphosphate modification, therefore the location of phosphotyrosine residues can be readily determined by the mass difference between two successive fragment ions of 243 Da. Indeed, the characteristic 'reporter' immonium ion at 216.043 Da can be used for precursor ion experiments in positive ion mode for selective identification of phosphotyrosine containing peptides [Steen H., Kuster B., Fernandez M., Pandey A., and Mann M. 2001. *Anal. Chem.* **73**: 1440-1448].

A common way to overcome the lability of the phosphoserine and phospho-threonine side chains during MS/MS is to replace the phosphate group with a more stable, less acidic side chain functionality. This can be accomplished by β–elimination of phosphoserine and phosphothreonine residues under strongly alkaline conditions to yield dehydroalanine or dehydroaminobutyric acid residues, respectively. Subsequent Michael addition of a nucleophile allows a simple means for derivatizing the formerly phosphorylated serine or threonine residues prior to mass spectrometric analysis [Weckwerth, W., Willmitzer, L. and Fiehn, O. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1677-1681.; Molloy, M.P. and Andrews, P.C. *Anal. Chem.*

2001, 73, 5387-5394.; Li, W., Boykins, R.A., Backlund, P.S., Wang, G. and Chen, H-C. *Anal. Chem.* **2002** *In Press.;* Steen, H. and Mann, M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 996-1003.]. The method can also be used to incorporate an affinity "tag" to allow enrichment [Adamczyk, M., Gebler, J.C. and Wu, J. *Rapid Commun Mass Spectrom.* **2001**, *15*, 1481-1488.; Oda, Y., Nagasu, T. and Chait, B.T. *Nature Biotechnol.* **2001**, *19*, 379-382.] of the derivatized peptides prior to their analysis and, coupled with the incorporation of an isotopic label, for quantitation of the degree of phosphorylation observed between two different sample sets [Goshe M.B., Conrads, T.P., Panisko, E.A., Angell, N.H., Veenstra, T.D. and Smith, R.D. *Anal Chem.* **2001**, *73*, 2578-2586.; Goshe, M.B.,Veenstra, T.D., Panisko, E.A., Conrads, T.P., Angell, N.H. and Smith, R.D. *Anal. Chem*, **2002**, *74*, 607-616.; Adamczyk, M; Gebler, J.C. and Wu, J. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 999-1001.].

Note that β-elimination affects both O-phospho and O-glycosidic linkages to the same extent. Therefore the β-elimination method cannot distinguish between O-linked post translational modifications of serine and threonine residues.

Another chemical method for analyzing the phosphoproteome, which is applicable to phosphotyrosine-containing peptides as well as those containing phosphoserine and phosphothreonine residues, has recently been described [Zhou, H., Watts, J.D. and Aebersold, R. *Nature Biotechnol* **2001**, *19*, 375-378]. For a recent review of quantitative phosphoproteome analysis see [Mann, M., Ong, S-E., Gronborg, M., Steen, H., Jensen, O.N. and Pandey, A. *Trends Biotechnol.* **2002**, 20, 261-268.].

Determination of protein-protein interactions are a crucial aspect of integrated biological studies aimed at understanding the complex pathways involved in cellular signaling and protein trafficking. Already, a large proportion of known protein-protein interactions in yeast [Bader, G.D. and Hogue, C.W.V. *Nature Biotechnol.* 2002, 20, 991-997] have been identified by genome-scale yeast two-hybrid assays [Uetz, P. *et al. Nature* **2000**, *403*, 623-627.; Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. and Sakaki, Y. *Proc. Natl. Acad. Sci. USA*, **2001**, *98*, 4569-4574.; Legrain, P. *Nature. Biotechnol.* **2002**, *20*, 128-129.], and direct affinity capture methods such as (i) co-precipitation using affinity-tagged recombinant proteins [Gavin, A-C. *et al. Nature* **2002**, *415*, 141-147.; Ho, Y. *et al. Nature.* **2002**, *415*, 180-183.], (ii) 'pull down' techniques using antibodies directed against one of the component proteins, (iii) protein-

affinity-interaction chromatography (e.g., using recombinant glutathione S-transferase (GST)-fusion proteins and glutathione-affinity chromatography), or (iv) isolation of intact multiprotein complexes (e.g., nuclear pore complexes, ribosome complexes, and spliceosomes). These later methods, coupled with mass spectrometry for protein identification, constitute the major methods for cell mapping proteomics, which aims to describe all protein-protein interactions (both spatially and temporally) within a given cell [Blackstock, W.P. and Weir, M.P. *Trends Biotechnol.* **1999**, *17*, 121-127.]. However, the interactions detected by these physical methods may include large numbers of non-specific interactions with no biological significance [Bader, G.D. and Hogue, C.W.V. *Nature Biotechnol.* **2002**, *20*, 991-997.].

Mass spectrometry combined with cross-linking [Rappsilber, J., Siniossoglou, S., Hurt, E. C., and Mann, M. *Anal. Chem.* **2000**, 72, 267-275.], or hydrogen/deuterium exchange [Yamada, N., Suzuki, E., and Hirayama, K. *Rapid Commun. Mass Spectrom.* **2002**, 16, 293-299] can be used for the rapid low-resolution evaluation of the three dimensional structures of proteins and protein complexes. Cross-linking generally involves chemical [Uy, R., and Wold, F., **1977**, In: "Protein Cross-linking" (Friedman, M., ed.), Plenum, New York.; Fancy, D.A. *Current Opin. Chem. Biol.* **2000**, *4*, 28-33.] or photochemical [Chowdhry, V., and Westheimer, F.H., *Annu. Rev. Biochem.* **1979**, 48, 293-325.; Fancy, D.A. and Kodadek, T. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6020-6024.] cross-linking of an isolated protein complex, followed by proteolytic digestion and MS and/or MS/MS analysis of the resulting peptide mixture, to subsequently locate proximally adjacent regions of the proteins being examined. However, the large number of peptide species that are generated following digestion of cross-linked proteins makes it often difficult to rapidly and unambiguously identify those peptides that are cross-linked. A number of different groups have developed methods to partially overcome this limitation, either by prior reduction of thiol cleavable cross-linkers [Bennett, K.L., Kussmann, M., Bjork, P., Godzwon, M., Mikkelsen, M., Sorensen, P. and Roepstorff, P. *Protein Sci.* **2000**, *9*, 1503-1518.], by using isotopic labelling methodologies [Chen, X., Chen, Y. H., and Anderson, V. E. *Anal. Biochem* **1999**, *273*, 192-203.; Pearson, K.M., Panell, L.K. and Fales, H.M. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 149-159.; Muller, D. R., Schindler, P., Towbin, H., Wirth, U., Voshol, H., Hoving, S., and Steinmetz, M.O. *Anal. Chem.* **2001**, *73*, 1927-1934.; Back, J.W., Notenboom, V., de Koning, L.J., Muijsers,

A.O., Sixma, T.K., de Koster, C.G., and de Jong, L. *Anal. Chem.* **2002**, *74*, 4417-4422.; Taverner, T., Hall, N.E., O'Hair, R.A.J. and Simpson, R.J. *J. Biol. Chem.* **2002** *In press*], or via the incorporation of a labile MS/MS "tag" on the cross linker itself [Back, J.W., Hartog, A.F., Dekker, H.L., Muijsers, A.O., de Koning, L.J. and de Jong, L. *J. Am. Soc. Mass Spectrom.* **2001**, 12, 222-227.].

It is important to recognise that all of the techniques described above for selective identification and differential quantitation based on incorporation of a differential isotopic "signature" rely on a common approach for peptide identification. That is, the isotopic signatures of the peptide ions of interest are detected by mass analysis of their intact precursor ions. Thus, limitations of the approach are encountered when; (i) ions of interest are present at low levels (i.e., approaching or below the level of chemical noise present in the spectrum) [Krutchinsky, A.N. and Chait, B.T. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 129-134.], (ii) the masses of low abundance differentially labeled peptides overlap with other higher abundance components present in the mixture, (iii) separation of the differential labelled "heavy" and "light" peptides occurs during chromatographic fractionation of the peptide mixture [Zhang, R.; Sioma, C.S.; Wang, S.; Regnier, F.E. *Anal. Chem.* **2001**, *73*, 5142-5149. Zhang, R., Sioma, C.S.; Thompson, R.A.; Xiong, L.; Regnier, F.E. *Anal. Chem.* **2002**, *74*, 3662-3669.; Zhang, R. and Regnier, F.E. *J. Proteome Res.* **2002**, 1, 139-147], or (iv) the mass spectrometer lacks sufficient resolution to adequately resolve the two labelled components, thereby precluding their detection.

Tandem mass spectrometry (MS/MS) dissociation methods [Mcluckey, S.A. and Goeringer, D.E. *J. Mass Spectrom.* **1997**, *32*, 461-474.] methods, whereby a precursor ion of interest is mass selected, subjected to fragmentation via collision-induced dissociation (CID) and then the resultant product ions mass analyzed to derive structural information relating to the amino acid sequence of the peptide or to indicate the presence and location of post-translational modifications, may be used to address at least some of the limitations indicated above. Due to the reduction in chemical noise associated with the formation of product ions at different m/z values to that of the mass selected precursor ion, low abundance ions that may not otherwise be apparent in the mass spectrum (i.e., below the level of chemical noise) may be successfully detected and analysed by MS/MS methods. Indeed, operation of a tandem mass spectrometer in precursor ion [Schwartz, J. C.; Wade, A. P.; Enke, C. G.; Cooks, R. G. *Anal.Chem.* **1990**, *62*, 1809-1818.; Schlosser A.,

Pipkorn R., Bossemeyer D., and Lehmann W.D. 2001. *Anal. Chem.* **73**: 170-176.; McClellan, J. E.; Quarmby, S. T.; Yost, R. A.; *Anal. Chem.* **2002**, In Press] or neutral loss [Schwartz, J. C.; Wade, A. P.; Enke, C. G.; Cooks, R. G. *Anal.Chem.* **1990**, *62*, 1809-1818.; Wilm M; Neubauer G; Mann M. *Anal. Chem.* **1996**, *68*, 527-33.; McClellan, J. E.; Quarmby, S. T.; Yost, R. A.; *Anal. Chem.* **2002**, In Press] MS/MS scan modes, whereby the mass spectrometer is set to detect diagnostic low mass product ions, or product ions offset by a given mass from the precursor ion, respectively, has been demonstrated to increase sensitivity by 1-2 orders of magnitude over conventional MS based detection methods [Wilm M; Neubauer G; Mann M. *Anal. Chem.* **1996**, *68*, 527-533.]. These precursor ion or neutral loss scan modes can be used to indicate the presence of peptides containing certain structural features via formation of diagnostic "signature ions" formed upon CID MS/MS, giving greater specificity compared to that of MS based methods, and overcoming the problem of low abundance peptides overlapping with other higher abundance components present in the mixture. One of the limitations of MS/MS based approaches however, is that fragmentation giving rise to the product ion or neutral loss of interest is usually only one of many dissociation channels, thereby "diluting" the spectrum, limiting sensitivity and potentially complicating subsequent interpretation of the spectra. Also, MS/MS methods for quantitation of differential protein expression have not been described.

We have developed a novel strategy for protein identification, differential quantitation and for the analysis of post translational modification status and cross-linking, involving the fixed-charge derivatization of selected peptides containing certain structural features, (eg the side chains of selected amino acids, or those containing post translational modifications or cross linking agents), that is, in its preferred forms, capable of addressing all of the MS and MS/MS limitations discussed above, with an expected increase in selectively and sensitivity of several orders of magnitude over the existing MS based approaches.

Derivatization strategies for mass spectrometric analysis are commonplace and have been reviewed previously [Knapp, D.R. *Methods Enzymology* **1990**, *193*, 314-329.; Anderegg, R.J. *Mass Spectrom. Rev.* **1988**, *7*, 395-424.; Roth, K.D.W., Huang, Z-H., Sadagopan N, and Watson J.T. *Mass Spectrom. Rev.* **1998**, *17*, 255-274.; Sadagopan, N. and Watson J.T. *J. Am. Soc. Mass. Spectrom.* **2001**, *12*, 399-409.; Jones, M.B., Jeffrey, W.A., Hansen,

H.F., Pappin, D.J.C. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 737-42.; Spengler, B., Luetzenkirchen, F., Metzger, S., Chaurand, P., Kaufmann, R., Jeffery, W., Bartlet-Jones, M. and Pappin, D.J.C. *Int. J. Mass Spectrom. Ion Proc.* **1997**, *169/170*, 127-140.; Keogh, T., Lacey, M.P., and Youngquist, R.S. *Rapid. Commum. Mass Spectrom.* **2000**, *14*, 2348.]. Although fixed charge derivatives of peptides have been used in conjunction with tandem mass spectrometry for sequencing applications, previous work has entirely focused on directing fragmentation toward the formation of a particular series of backbone cleavage derived sequence ions (i.e., maximizing sequence coverage), and has been limited to the derivatization of the N- and C-termini, as well as lysine and arginine side chains.

In contrast, the present invention is based on a fixed-charge derivatization approach, which is designed to either (i) direct the dissociation of the peptide toward a single predictable fragmentation channel, resulting in the formation of a single product, or (ii) to enable fixed-charge derivative containing peptides to undergo incomplete charge neutralization during an ion-ion proton transfer reaction period [Reid, G.E., Wells, J.M., Badman, E.R. and McLuckey S.A. *Int. J. Mass Spectrom.* **2002**, *In Press.*; Reid, G.E.; Shang, H.; Hogan, J.M.; Lee, G.U.; McLuckey, S.A.. *J. Am. Chem. Soc.* **2002**, *124*, 7353-7362.; McLuckey, S.A.; Reid, G.E.; Wells, J.M. *Anal. Chem.* **2002**, *74*, 336-346.; McLuckey, S.A.; Stephenson, J.L. *Mass Spectrom. Rev.* **1999**, *17*, 369-407.; Stephenson, J.L.; Mcluckey, S.A. *J. Mass Spectrom.* **1998**, *33*, 664-672.; Stephenson, J.L; McLuckey, S.A. *Anal. Chem.* **1998**, *70*, 3533-3544.; Stephenson, J.L.; Mcluckey, S.A. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 585-596.; McLuckey, Scott A.; Stephenson, James L.; Asano, Kelji G. *Anal. Chem.* **1998**, *70*, 1198-1202.; Stephenson, James L.; McLuckey, Scott A.. *Int. J. Mass Spectrom. Ion Proc.* **1997**, *165/166*, 419-431.; Stephenson, J.L.; McLuckey, S.A. *Anal. Chem.* **1997**, *69*, 3760-3766.; Stephenson, J.L..; McLuckey, S.A. *J. Am. Chem. Soc.* **1996**, *118*, 7390-7397.], thereby allowing its selective identification from a complex mixture, then automatically subjecting it to further structural interrogation, using MS/MS or multistage MS/MS (MS^n), or by determination of an "accurate mass tag" of the precursor ion [Conrads, T.P., Anderson, G.A., Veenstra, T.D., Pasa-Tolic, L. and Smith, R.D. *Anal. Chem.* **2000**, *72*, 3349-3354.; Goodlet, D.R., Bruce, J.E., Anderson, G.A., Rist, B., Pasa-Tolic, L., Fiehn, O., Smith, R.D. and Aebersold, R. *Anal. Chem.* **2000**, *72*, 1112-1118.], to enable its characterization.

The general principles behind the use of the fixed-charge peptide ion derivatization approach for directed CID MS/MS fragmentation are demonstrated here for the selective identification and differential quantitation of side chain fixed-charge sulfonium ion derivatives of peptides containing methionine (fixed-charge sulfonium ion derivatives of the amino acids methionine and cysteine, which were observed to fragment exclusively via neutral loss of the side chain CH_3SR, where R was a substituted alkyl group [Reid, G.E., Simpson, R.J. and O'Hair, R.A.J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 1047-1060.; O'Hair, R.A.J. and Reid, G.E. *Eur. Mass Spectrom.* **1999**, *5*, 325–334.], have previously been employed for the independent gas-phase synthesis of potential product ions involved in the side chain fragmentation reactions of amino acids.). Comparable strategies for the selective analysis of peptides containing other amino acids, those that contain post translationally modified amino acids, as well as for the characterization of cross-linked peptides are also described.

SUMMARY OF THE INVENTION

In a first embodiment, the present invention provides a method of analysis of proteins or peptides, the method comprising:

(1) passing a mixture of proteins or peptides containing at least one protein or peptide derivatized with a fixed-charge ion, other than at the C-terminal or N-terminal end thereof, through a first mass resolving spectrometer to select precursor protein or peptide ions having a first desired mass-to-charge ratio;

(2) subjecting the precursor ions of the first mass to charge ratio to dissociation to cause selective fragmentation of the peptide to form a product ion having a characteristic second mass-to-charge ratio; and

(3) detecting the product ions.

The product ion having the second characteristic mass-to-charge ratio may be either a charged protein or peptide containing product ion formed by *neutral* loss of the fixed charge from the precursor ion, or a product ion formed by *charged* loss of the fixed charge from the precursor ion.

Preferably, the method of the invention comprises the further step of:

(4) determining the identity of the peptide or protein.

Step (4) may be performed by first repeating steps (1) and (2) and then subjecting the product ion having the second characteristic mass-to-charge

ratio formed by (i) neutral loss from the precursor, which will have a charge state the same as that of the precursor, or (ii) the complementary product ion to the charged product ion formed by charged loss from the precursor ion, which corresponds to a protein or peptide containing product ion having a charge state one lower than the precursor, to a further stage of dissociation to form a series of product ions having a range of mass to charge ratios, for the purpose of determining the amino acid sequence of the peptide and subsequently, the identity of its protein of origin.

Alternatively, step (4) may be carried out by use of high resolution mass analyzers to obtain mass accuracies approaching 1 ppm on the product ion detected in step (3), or its complementary product ion (i.e., to derive an "accurate product ion mass tag"). This, coupled with database searching, may be employed for subsequent identification of those peptides found to contain a fixed charge derivative. Previously, in cases where an "accurate mass tag" has been obtained for a precursor ion, and the presence of a particular amino acid is known (for example the presence of a cysteine residue), the specificity of database searching algorithms can be improved such that unambiguous identification of the protein from which the peptide is derived has been achieved from this information alone.

The protein or peptide ion may be dissociated by any suitable dissociation method including, but not limited to, collisions with an inert gas (known as collision-induced dissociation (CID or collisionally-activated dissociation (CAD); (ii) collisions with a surface (known as surface-induced dissociation or SID); (iii) interaction with photons (e.g. via a laser) resulting in photodissociation; (iv) thermal/black body infrared radiative dissociation (BIRD), and (v) interaction with an electron beam, resulting in electron-induced dissociation for singly charged cations (EID), or electron-capture dissociation (ECD) for multiply charged cations.

In a second embodiment, the present invention provides a method of analysis of proteins or peptides, the method comprising:

(1) introducing a mixture of proteins or peptides containing at least one protein or peptide derivatized with a fixed-charge ion, other than at the C-terminal or N-terminal end thereof, into a mass spectrometer;

(2) subjecting the ions to charge neutralization reactions using ion-ion proton transfer reactions; and

(3) detecting the remaining product ions.

The remaining product ions may be those protein or peptide ions that contain singly charged fixed charge derivatives, and may have a mass to charge ratio the same as the precursor, if the initial charge state was one, or a new mass to charge ratio if the charge state of the precursor was greater than one.

The method of the second embodiment may comprise the further step of:

(4) determining the identity of the peptide or protein.

Step (4) of the second embodiment may be performed by first repeating steps (1) and (2) and then subjecting the remaining product ions to dissociation by the methods described in respect steps (2,3 and 4) of the first embodiment as described above.

The method of analysis of the present invention may be used for protein/peptide identification, differential quantitation, analysis of post translational modification status or analysis of cross-linking status.

The present invention provides a method of analysis of a protein or peptide containing a fixed charge derivative, at a site other than at the C-terminal or N-terminal end thereof.

The fixed-charge derivative may be contained on a side-chain of a selected amino acid residue contained within a protein or peptide. Preferably the selected amino acid residue is that of a "rare" amino acid, as described in more detail below. The fixed-charge derivative may be contained on a side-chain of a post translational modified amino acid residue, as described in more detail below. The fixed-charge derivative may be on a cross-link contained between two proteins or peptides, as described in more detail below.

Non-limiting examples of the fixed-charge include a sulfonium ion, a quaternary alkylammonium or a quaternary alkylphosphonium ion.

The selected amino acid residue may be one containing a S atom in the side chain thereof. The side chain may also contain an S-alkyl group. Preferred amino acid residues are methionine, cysteine and S-alkyl cysteine. The selected amino acid residue may also be tryptophan or tyrosine or histidine. Derivatization of the side chain of a selected amino acid residue to introduce a fixed-charge may be achieved by strategies known in the art.

The fixed-charge may also be contained on an O-linked post-translationally modified amino acid residue (for example, a dehydroalanine

residue formed by β-elimination from a post-translationally modified serine amino acid residue, or a dehydroamino-2-butyric acid residue formed by β-elimination from a post-translationally modified threonine amino acid residue) contained within a protein or peptide. Derivatization of a formerly post-translationally modified amino acid residue to introduce a fixed-charge may be achieved by strategies known in the art.

The fixed-charge may also be contained within a cross-linking reagent or a cross-link contained between two proteins or peptides. Derivatization of a cross-linking reagent or a cross-link contained between two proteins or peptides to introduce a fixed-charge may be achieved by strategies known in the art.

Analysis of the protein or peptide ion may be performed by tandem mass spectrometry. The tandem mass spectrometer may be equipped with electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI) interfaces to transfer the protein or peptide ion from solution into the gas-phase. Mass analysers that are applicable to tandem mass spectrometry fall into two basic categories: tandem-in-space and tandem-in-time. Tandem-in-space mass spectrometers have discreet mass analysers for each stage of mass spectrometry; examples include sector (commonly double focusing sector and "hybrid" combinations of sector and quadrupole analyser instruments), time of flight and triple quadrupole instruments, as well as "hybrid" combinations of time of flight and quadrupole instruments. Tandem-in-time mass instruments have only one mass analyser, and each stage of mass spectrometry takes place in the same region, but is separated in time via a sequence of events. Examples of tandem in time mass analysers include both two- and three-dimensional quadrupole ion trap and Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometers.

The fixed-charge derivatization approach in the method of the present invention may be applied to the quantitation of differential protein expression based on the incorporation of suitable isotopic (eg., ^{13}C, ^{15}N, ^{2}H)) or structural labels to the fixed charge. The fixed-charge derivatization approach in the method of the present invention may be also applied to the identification and quantitation of post translational modification status in proteins by incorporation of the fixed-charge derivative, via the β-elimination/Michael addition method for forming mass spectrometry stable derivatives of O-phosphorylated and O-glycosylated amino serine, or O-phosphorylated and O-glycosylated threonine,

for example, that is described in Meyer, H., Hoffman-Posorske, E., Korte, H. and Heilmeyer, L.J. *FEBS. Lett.* **1986**, *204*, 61-66., the disclosure of which is incorporated herein by reference. The fixed-charge derivatization approach in the method of the present invention may also be applied to the identification and characterization of protein-protein interactions via incorporation of the fixed-charge derivative into a suitable cross-linking reagent.

As already mentioned above, in the method of the first embodiment, tandem mass spectrometry of the fixed-charge derivatized peptides results in exclusive formation of a product ion via fragmentation at the fixed-charge site upon dissociation, or in the method of the second embodiment, results in incomplete charge neutralization of fixed charge derivative containing peptides upon ion-ion reactions, thereby allowing specific identification of only those peptides containing the derivatization, without need for prior resolution or otherwise enrichment of the complex mixture prior to analysis.

The method of the present invention, in one particular embodiment, will be described below with reference to the selective identification and differential quantitation of peptides present at a range of different ratios and by the incorporation of isotopically labelled versions of the fixed-charge derivative, to yield a 'light' form (containing only natural isotopes), and a 'heavy' form (containing isotopic or structural labels incorporated into the substituent), with selective identification and differential quantitation of peptide levels performed by neutral loss mode tandem mass spectrometry. Application of the general approach to the analysis of post translational modification status as well as selective identification of protein-protein interactions by cross-linking analysis are also described.

The present invention will now be described with reference to particular embodiments, however, the method of the present invention is not limited to these particular embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Quadrupole ion trap tandem mass spectrometry of singly charged GAILMGAILA and its methionine side chain fixed-charge derivative. (A) CID MS/MS of the $[M+H]^+$ ion. (B) (inset) CID MS/MS of the methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)]^+$ ion. (main) CID MS^3 of the $[M-CH_3S(AP)]^+$ product ion in Figure 1B(inset).

Figure 2. Quadrupole ion trap tandem mass spectrometry of doubly charged GAILMGAILA and its methionine side chain fixed-charge derivative. (A) CID MS/MS of the $[M+2H]^{2+}$ ion. (B) (inset) CID MS/MS of the methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ion. (main) CID MS^3 of the $[M-CH_3S(AP)]^{2+}$ product ion in Figure 2B(inset).

Figure 3. Quadrupole ion trap tandem mass spectrometry of singly charged GAILMGAILK and its methionine side chain fixed-charge derivative. (A) CID MS/MS of the $[M+H]^+$ ion. (B) (inset) CID MS/MS of the methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)]^+$ ion. (main) CID MS^3 of the $[M-CH_3S(AP)]^+$ product ion in Figure 3B(inset).

Figure 4. Quadrupole ion trap tandem mass spectrometry of doubly charged GAILMGAILK and its methionine side chain fixed-charge derivative. (A) CID MS/MS of the $[M+2H]^{2+}$ ion. (B) (inset) CID MS/MS of the methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ion. (main) CID MS^3 of the $[M-CH_3S(AP)]^{2+}$ product ion in Figure 4B(inset).

Figure 5. Quadrupole ion trap tandem mass spectrometry of singly charged GAILMGAILR and its methionine side chain fixed-charge derivative. (A) CID MS/MS of the $[M+H]^+$ ion. (B) (inset) CID MS/MS of the methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)]^+$ ion. (main) CID MS^3 of the $[M-CH_3S(AP)]^+$ product ion in Figure 5B(inset).

Figure 6. Quadrupole ion trap tandem mass spectrometry of doubly charged GAILMGAILR and its methionine side chain fixed-charge derivative. (A) CID MS/MS of the $[M+2H]^{2+}$ ion. (B) (inset) CID MS/MS of the methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ion. (main) CID MS^3 of the $[M-CH_3S(AP)]^{2+}$ product ion in Figure 6B(inset).

Figure 7. Quadrupole ion trap tandem mass spectrometry of the triply charged methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivatives of GAILMGAILK and GAILMGAILR. (A) (inset) CID MS/MS of the $[M(AP)+2H]^{3+}$ ion of GAILMGAILK. (main) CID MS^3 of the $[M-CH_3S(AP)]^{3+}$ product ion in Figure 7A(inset). (B) (inset) CID MS/MS of the $[M(AP)+2H]^{3+}$ ion of GAILMGAILR. (main) CID MS^3 of the $[M-CH_3S(AP)]^{3+}$ product ion in Figure 7B(inset).

Figure 8. Energy resolved CID MS/MS of the doubly charged methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ion of GAILMGAILK.

Figure 9. Individual CID MS/MS product ion spectra of the doubly charged methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ion of GAILMGAILK from Figure 8. (A) Product ion spectrum obtained at a collision energy of 12V. (B) Product ion spectrum obtained at a collision energy of 22V.

Figure 10. Energy resolved CID MS/MS of the singly charged methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)]^{+}$ ions of GAILMGAILA, GAILMGAILK and GAILMGAILR.

Figure 11. Energy resolved CID MS/MS of the doubly charged methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ions of GAILMGAILA, GAILMGAILK and GAILMGAILR.

Figure 12. Energy resolved CID MS/MS of the triply charged methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+2H]^{3+}$ ions of GAILMGAILK and GAILMGAILR.

Figure 13. Energy resolved CID MS/MS of the $[M+H]^{+}$ and $[M+2H]^{2+}$ ions of GAILMGAILA, GAILMGAILK, GAILMGAILR, GAILAGAILA, GAILAGAILK and GAILAGAILR. (A) $[M+H]^{+}$ and $[M+2H]^{2+}$ ions of

GAILMGAILA, GAILMGAILK and GAILMGAILR. (B) $[M+H]^+$ and $[M+2H]^{2+}$ ions of GAILAGAILA, GAILAGAILK and GAILAGAILR

Figure 14. Mass spectrum of a 1:1 mixture of d_0 and d_5 containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivative of GAILMGAILR.

Figure 15. Neutral loss tandem mass spectrometry of the 1:1 d_0 and d_5 containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivatives of GAILMGAILR from Figure 14. (A) Neutral loss CID MS/MS spectra of the doubly charged d_0- and d_5- containing $[M(AP)+H]^{2+}$ ions (83 Da and 85.5 Da, respectively). (B) Neutral loss CID MS/MS spectra of the triply charged d_0- and d_5- containing $[M(AP)+2H]^{3+}$ ions (55.3 Da and 57 Da, respectively).

Figure 16. Individual CID MS/MS product ion spectra of the doubly charged d_0- containing methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+H]^{2+}$ ion of GAILMGAILR from Figure 15. (A) Product ion spectrum obtained at a collision energy of 17.5V. (B) Product ion spectrum obtained at a collision energy of 31V.

Figure 17. Individual CID MS/MS product ion spectra of the triply charged d_0-containing methionine side chain fixed-charge acetophenone (AP) sulfonium $[M(AP)+2H]^{3+}$ ion of GAILMGAILR from Figure 15. (A) Product ion spectrum obtained at a collision energy of 13V. (B) Product ion spectrum obtained at a collision energy of 18V.

Figure 18. Mass spectrum of a 2:1 mixture of d_0 and d_5 containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivative of GAILMGAILR.

Figure 19. Neutral loss tandem mass spectrometry of the 2:1 d_0 and d_5 containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivatives of GAILMGAILR from Figure 18. (A) Neutral loss CID MS/MS spectra of the doubly charged d_0- and

d_5- containing $[M(AP)+H]^{2+}$ ions (83 Da and 85.5 Da, respectively). (B) Neutral loss CID MS/MS spectra of the triply charged d_0- and d_5- containing $[M(AP)+2H]^{3+}$ ions (55.3 Da and 57 Da, respectively).

Figure 20. Mass spectrum of a 5:1 mixture of d_0 and d_5 containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivative of GAILMGAILR.

Figure 21. Neutral loss tandem mass spectrometry of the 5:1 d_0 and d_5 containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivatives of GAILMGAILR from Figure 20. (A) Neutral loss CID MS/MS spectra of the doubly charged d_0- and d_5- containing $[M(AP)+H]^{2+}$ ions (83 Da and 85.5 Da, respectively). (B) Neutral loss CID MS/MS spectra of the triply charged d_0- and d_5- containing $[M(AP)+2H]^{3+}$ ions (55.3 Da and 57 Da, respectively).

EMBODIMENTS OF THE INVENTION

Definitions

Unless the context indicates otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood in the art to which the present invention belongs.

"Fixed-charge", as used herein, includes any charge localised to a specific heteroatom contained within the protein or peptide, or to a specific heteroatom contained within the derivatization reagent (eg in solution or in the gas-phase), by the attachment of any moiety.

"Fixed charge derivatization", as used here, means the introduction of a fixed charge as defined above. For example, the fixed charge may be introduced either by introducing a neutral reagent to subsequently form the fixed charge at a specific site within the protein or peptide, or by introduction of a reagent containing the fixed charge to a specific site within the protein or peptide.

In respect to the first embodiment of the present invention, the fixed charge derivative thus formed preferably has a structure such that it allows the exclusive formation of a product ion upon dissociation that is characteristic of the fixed charge derivative. In respect to the second embodiment of the present invention, the fixed charge derivative preferably has a structure such that it is non-reactive to charge neutralization by proton transfer or group transfer reactions during ion-ion reactions.

"Protein", as used herein, means any protein, including, but not limited to peptides, enzymes, glycoproteins, hormones, receptors, antigens, antibodies, growth factors, etc., without limitation. Proteins may be endogenous, or produced from other proteins by chemical or proteolytic cleavage. Preferred proteins include those comprised of at least 15-20 amino acid residues. The term includes cross-linked proteins.

"Peptide" as used herein includes any substance comprising two or more amino acids and includes di-, tri-, oligo and polypeptides etc according to the number of amino acids linked by amide(s) bonds. Peptides may be endogenous, or produced from other peptides or proteins by chemical or proteolytic cleavage. Preferred peptides include those comprised of up to 15-20 amino acid residues. The term includes cross-linked peptides.

When the amino acids are α-amino acids, either the L-optical isomer or the D-optical isomer can be used. The L -isomers are generally preferred. For a general review, see, Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

The term "alkyl " is used herein to refer to a branched or unbranched, saturated or unsaturated, monovalent hydrocarbon radical. The hydrocarbon radical may have from about 1-20 carbons and preferably, from 3-15 carbons, and more preferably from 3-10 carbons. When the alkyl group has from 3-10 carbon atoms, it is referred to as a "lower alkyl." Suitable alkyl groups include, for example, structures containing one or more methylene, methine and/or methyne groups. Branched structures have a branching motif similar to i-propyl, t-butyl, i-butyl, 2-ethylpropyl, etc. As used herein, the term encompasses "substituted alkyls," and "cyclic alkyl."

"Substituted alkyl" refers to alkyl as just described including one or more substituents such as, for example, lower alkyl, aryl, acyl, halogen (i.e., alkylhalos, e.g., CF_3), hydroxy, amino, amide, alkoxy, alkylamino, acylamino, thioamido, acyloxy, aryloxy, aryloxyalkyl, ether, ester, disulfide, mercapto, thia, aza, oxo, both saturated and unsaturated cyclic hydrocarbons, heterocycles and the like. These groups may be attached to any carbon or substituent of the alkyl moiety. Additionally, these groups may be pendent from, or integral to, the alkyl chain. For the purpose of covalent solid phase pre-enrichment of fixed charge derivatives, the substituted alkyl group may be covalently attached to an insoluble bead or polymer, and contain a chemical or photochemical cleavage site between the insoluble bead or polymer and the alkyl group.

The term "aryl" is used herein to refer to an aromatic substituent, which may be a single aromatic ring or multiple aromatic rings which are fused together, linked covalently, or linked to a common group such as a methylene or ethylene moiety. The common linking group may also be a carbonyl as in benzophenone. The aromatic ring(s) may include phenyl, naphthyl, biphenyl, diphenylmethyl and benzophenone among others. The term "aryl" encompasses "arylalkyl" and "substituted aryl."

"Substituted aryl" refers to aryl as just described including one or more functional groups such as lower alkyl, acyl, halogen, alkylhalos (e.g. CF_3), hydroxy, amino, alkoxy, alkylamino, acylamino, acyloxy, phenoxy, mercapto and both saturated and unsaturated cyclic hydrocarbonswhich are fused to the

aromatic ring(s), linked covalently or linked to a common group such as a methylene or ethylene moiety. The linking group may also be a carbonyl such as in cyclohexyl phenyl ketone. The term "substituted aryl" encompasses "substituted arylalkyl."

The term "arylalkyl" is used herein to refer to a subset of "aryl" in which the aryl group is attached to another group by an alkyl group as defined herein.

"Substituted arylalkyl" defines a subset of "substituted aryl" wherein the substituted aryl group is attached to another group by an alkyl group as defined herein.

The term "acyl" is used to describe a ketone substituent, –(O)R, where R is alkyl or substituted alkyl, aryl or substituted aryl as defined herein.

Abbreviations

CID	Collision Induced Dissociation
ESI	Electrospray Ionization
MALDI	Matrix Assisted Laser Desorption Ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multistage tandem mass spectrometry, where n >2

Materials and Methods.

Materials

The model synthetic peptides (GAILMGAILA, GAILMGAILK, GAILMGAILR, GAILAGAILA, GAILAGAILK and GAILAGAILR) were obtained from Auspep (Melbourne, Vic, Australia) and used directly without further purification. The alkylating reagents, iodomethane, iodoethane, iodobenzene, iodomethylbenzene, d_5-acetophenone and bromoacetophenone were purchased from Aldrich (Castle Hill, NSW, Australia). Polymer supported pyridyl bromide perbromide, iodoacetic acid and iodoacetamide were from Sigma (St. Louis, MI, USA). Methanol, and acetonitrile (Chromar grade) were purchased from Mallinkrodt (Paris, KY, USA). Formic acid and acetic acid were obtained from BDH Laboratories (Poole, England). All solutions were prepared using deionised water purified by a tandem Milli-Q and Milli-RO system (Millipore, Bedford, MA, USA).

Synthesis of d_5-bromoacetophenone.

d_5-bromoacetophenone was synthesized according to the method of Frechet *et al* [Frechet, J.M.J., Farrall, M.J. and Nuyens, L.J. *J. Macromol. Sci.-Chem.* **1977**, *A11*, 507-514.] Briefly, 1.25 mL of d_5-acetophenone was added to 4.8 g Polymer supported pyridyl bromide perbromide (~3 meq Br_3^- /g resin) in 30 mL methanol then allowed to react with stirring at room temperature for 4 hours. The resultant α-bromo ketone was obtained in pure form by filtration of the reaction mixture and evaporation of the solvent, followed by recrystallization prior to use.

Side chain fixed-charge derivatization of methionine containing peptides.

Side chain fixed-charge sulfonium ion derivatives of methionine-containing peptides were produced by the addition of 10 µL of a 1M solution of alkylating reagent to 100 µg of peptide dissolved in 100 µL of aqueous 20% acetic acid containing 30% CH_3CN. The reaction was allowed to proceed for 16 hours at room temperature after which the sample was diluted and then introduced to the mass spectrometer with no further purification.

Mass Spectrometry

Mass spectrometric analysis was performed using either (i) quadrupole ion trap (Finnigan-MAT model LCQ-DECA, San Jose, CA), (ii) quadrupole-time-of-flight (Micromass model Q-TOF2, Manchester, UK), or (ii) triple quadrupole (Finnigan model TSQ, San Jose, CA) mass spectrometers, all equipped with electrospray ionization interfaces.

Quadrupole Ion Trap Mass Spectrometry.

Samples (10 pmol/µL in 50:50:1 H_2O:CH_3CN:acetic acid) were introduced to the mass spectrometer at 2 µL/min. The ESI conditions were optimized to maximize the intensity of the ion of interest. Typical conditions were: spray voltage –5 kV, Nitrogen sheath gas, 30 psi, heated capillary temperature 150°C. MS/MS and MS^3 experiments were performed on mass

selected ions using standard isolation and excitation procedures. All spectra collected were the average of 10 scans.

Quadrupole-time-of-flight mass spectrometry.

Spectra were acquired using the V-optics mode of the time-of-flight mass analyser following infusion of samples (same concentration as solution composition as for ion trap experiments) at 1 µL/min. Electrospray interface conditions were optimized to maximize the intensity of the ion of interest. Typical conditions were: spray voltage, –4.5 kV; nitrogen source gas, 1 psi; cone gas, 100 (arbitrary units); source temperature, 50°C; desolvation temperature, 150°C; cone voltage, 50V (singly charged ions), 30V (doubly charged ions) and 20V (triply charged ions). Energy resolved CID MS/MS experiments were automatically acquired on an isolated isotopic envelope precursor ion population to allow determination of product ion charge states, using argon as the inert collision gas at a pressure of 10 psi. The collision energy was ramped from 4 to 58 V in 1V increments for singly charged ions, and from 4 to 20 V in 1V increments and from 20 to 46V in 2V increments for doubly and triply charged ions. Spectra obtained for each collision energy value are the average of 10 scans.

Triple Quadrupole Mass Spectrometry.

Samples (same concentration as solution composition as for ion trap experiments) were introduced to the mass spectrometer by a home built nano-electrospray ionization source at a flow rate of 200 nL/min. The spray voltage was maintained at –1.8 kV. The heated capillary temperature was 150°C. The argon collision gas pressure was maintained at 1.5 mtorr. The instrument was operated at unit resolution. The instrument was operated under unit resolution conditions. Neutral loss mode MS/MS scans (neutral losses of 83 and 85.5 Da for d_0- and d_5- containing doubly charged ions, and 55.3 and 57 Da for d_0- and d_5- containing triply charged ions) were performed at collision energies of 18V and 13V, respectively. Product ion CID MS/MS spectra of peptide ions selectively identified by neutral loss scans were then acquired at 18V and 31V, and 13V and 18V for doubly and triply charged ions, respectively. All spectra shown are the average of 20 scans.

Results and Discussion

Identification of proteins by CID MS/MS of their fixed-charge derivative containing peptides.

Side chain fixed-charge derivatization of peptides containing methionine has been employed here to demonstrate the general strategy for selective identification and quantitation of peptides by selective CID MS/MS dissociation. The rationale for using fixed-charge derivatives of methionine containing peptides is based on the idea that approaches for selective identification and quantitation of differential protein expression should be specific for the detection of peptides containing amino acids that are rare, thereby limiting the number of peptides required to be analyzed, yet providing comprehensive coverage of proteins in the sequence databases. Release 40.0 of the SWISS-PROT database contains a total of 101,602 protein entries. The occurrence of methionine in this database is 2.37%. In contrast, the occurrence of cysteine, the amino acid targeted in the popular ICAT and related methods for selective mass spectrometric detection and quantitation, is 1.63%. Of the individual database entries, 85.33% contain cysteine while 96.9% contain methionine. 98.35% coverage could be attained if proteins containing both amino acids were targeted for analysis, as only 1.65% of entries do not contain either methionine or cysteine. Therefore, approaches directed toward the identification of proteins containing methionine (or both methionine and cysteine) should cover a significantly greater fraction of the proteins represented in the database than approaches targeting for cysteine alone.

The main strategy devised here for the selective identification and subsequent differential quantitation of methionine-containing peptides, as well as for S-alkyl cysteine, by fixed-charge sulfonium ion derivatization (i.e., derivatives in which the side chain of methionine or S-alkyl cysteine has been alkylated) and tandem mass spectrometry, are outlined below. A comparable strategy for the selective identification and differential quantitation of tryptophan (1.21% occurrence) or tyrosine (3.16% occurrence) containing peptides, as well as a strategy for the selective identification and differential quantitation of cysteine containing peptides, via quaternary alkylammonium ion derivatization

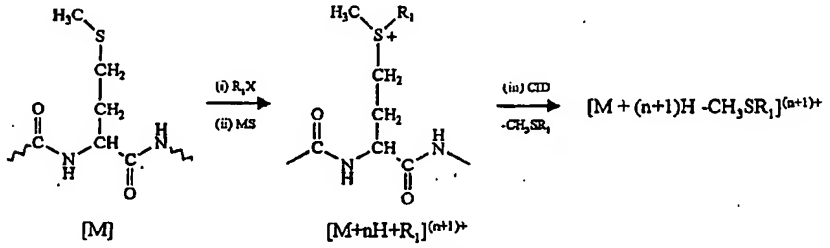
(with fragmentation directed toward the formation of a specific low mass product ion) are also discussed.

Fixed-charge sulfonium ion derivatization of peptides containing methionine.

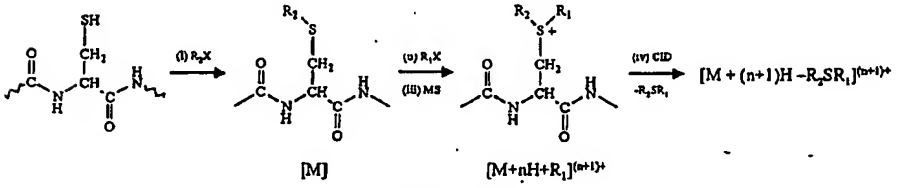
Identification of methionine-containing peptides via CID of its sulfonium ion fixed-charge derivative is attractive for several reasons. Methionine is a rare amino acid (see above). The chemistry and biological applications of sulfonium ions are well documented, giving precedent for developing reagents to form these ions in applications involving specific derivatization of peptides [Stirling, M.J. and Patai, S. The Chemistry of the Sulfonium Group, Wiley: New York, **1981**.; Lundblad. Techniques in Protein Modification, Chapter 8 "The Modification of Methionine" CRC Press. Florida, **1995**.; Liu T.-H. "The role of Sulfur in Proteins" In: The Proteins. Volume III, Neurath, Hill and Boeder. Eds., Academic NY, **1977**, Chapter 3, pp 239-402.; Gundlach, H.G. Moore, S, and Stein, W.H. *J. Biol. Chem.*, **1959**, *234*, 1761-1764.; Lawson, W.B. Gross, E., Foltz, C.M. and Witkop, B. *J. Am. Chem. Soc.*, **1961**, *83*, 1509-1510.; Lawson, W.B. Gross, E., Foltz, C.M. and Witkop, B. *J. Am. Chem. Soc.*, **1962**, *84*, 1715-1718.; Gross E. *Methods Enzymology* **1967**, *11*, 238-255.; Degen, J. and Kyte, *J. Anal. Biochem.* **1978**, *89*, 529-539.; Kyte, J., Degen, J. and Harkins, R.N. *Methods Enzymology* **1983**, *91*, 367-377.]. The alkylation of methionine residues in proteins and peptides is specific at low pH regardless of the alkylating reagent used [Lundblad. Techniques in Protein Modification, Chapter 8 "The Modification of Methionine" CRC Press. Florida, **1995**.; Liu T.-H. "The role of Sulfur in Proteins" In: The Proteins. Volume III, Neurath, Hill and Boeder. Eds., Academic NY, **1977**, Chapter 3, pp 239-402.]. The reason for this selectivity is that the rate of alkylation of the thioether sulfur atom of the methionine side chain is virtually independent of pH (even down to pH1) whereas the reactivity of all other nucleophilic functional groups (e.g. cysteine, lysine and histidine residues) decreases at low pH (due to protonation). Previously, sulfonium ions have been exploited to: (a) tag methionine sites in peptides and proteins [Lundblad. Techniques in Protein Modification, Chapter 8 "The Modification of Methionine" CRC Press. Florida, **1995**.; Liu T.-H. "The role of Sulfur in Proteins" In: The Proteins. Volume III, Neurath, Hill and Boeder. Eds., Academic NY, **1977**, Chapter 3, pp 239-402.; Gundlach, H.G. Moore, S, and Stein, W.H. *J. Biol. Chem.*, **1959**, *234*, 1761-1764.; Rogers, G.A. Shaltiel,

N, and Boyer, P.D. *J. Biol. Chem.*, **1976**, *251*, 5711-5717.; Toennies, G. and Kolb, J.J. *J. Am. Chem. Soc.*, **1945**, *67*, 849-851.; Lavine, T.F. Floyd, N.F, and Cammaroti, M.S. *J. Am. Chem. Soc.*, **1945**, *67*, 849-851.; Lawson, W.B. and Schramm, H.J. *J. Am. Chem. Soc.*, **1962**, *84*, 2017-2018.]; (b) to induce cleavage of peptide bonds adjacent to methionine residues via the solution phase fragmentation of the sulfonium ions. [Lawson, W.B. Gross, E., Foltz, C.M. and Witkop, B. *J. Am. Chem. Soc.*, **1961**, *83*, 1509-1510.; Lawson, W.B. Gross, E., Foltz, C.M. and Witkop, B. *J. Am. Chem. Soc.*, **1962**, *84*, 1715-1718.; Jendrek, J.P., Barker, R.H. and Altschul, A.M. *Biochim Biophys Acta*, **1967**, *136*, 409-411.; Gross E. *Methods Enzymology* **1967**, *11*, 238-255.] The most famous and widely studied system is the cyanogen bromide reaction [Gross E. *Methods Enzymology* **1967**, *11*, 238-255.], although other alkylating reagents have been used to similar effect [Tang, J.R. and Hartley, B.S. *Biochem. J.* **1967**, *102*, 593.; Tang, J.R. and Hartley, B.S. *Biochem. J.* **1970**, *118*, 611.]. Note that related reactions have been observed in peptide syntheses [Gairi, M., Lloyd-Williams, P., Albericio, F. and Giralt, E. *Tetrahedron Lett.* **1994**, *35*, 175-178.], as an autolytic protein cleavage reaction [Taylor, KL.; Pohl, J; Kinkade, J.M., *J. Biol. Chem.* **1992**, *267*, 25282-25288.], in the purification of peptides [Tang, J.R. and Hartley, B.S. *Biochem. J.* **1967**, *102*, 593.; Degen, J. and Kyte, J. *Anal. Biochem.* **1978**, *89*, 529-539.; Kyte, J., Degen, J. and Harkins, R.N. *Methods Enzymology* **1983**, *91*, 367-377.; Weinberger, S.R.; Viner, R.I. and Ho, P. *Electrophoresis.* **2002**, *23*, 3182-3192.], and to transform methionine residues to homocysteine residues [Chassaing, G; Lavielle, S; Marquet, A. *J. Org. Chem.* **1983**, *48*, 1757-60.]. Preliminary experiments involving sulfonium ions derivatives of methionine have been performed, using substituted alkyl halides R_1X (where X is the halogen and R is the substituted alkyl group) [O'Hair, R.A.J. and Reid, G.E. *Eur. Mass Spectrom.* **1999**, *5*, 325–334.]. The reagent used in these preliminary studies reacts specifically with the side chain of methionine in solution under acidic pH conditions to form a stable side chain sulfonium ion derivative ($-CH(CH_2)_2S(CH_3)R_1^+$) (step (i) of Scheme 1). Under these conditions, the reagent does not react with the free thiol containing cysteine. The reagent may however, be used for derivatization of cysteine-containing peptides after initial S-alkylation of the cysteine thiol side chain under basic pH conditions to yield the derivative ($-CHCH_2SR_2$) (step (i) of Scheme 2). Further alkylation of these S-alkyl cysteine containing peptides under identical

conditions to those employed for alkylation of methionine can then be used to form stable sulfonium ion derivatives of cysteine (-$CHCH_2SR_1R_2^+$) [Foti, S., Saletti, R. and Marletta, D. *Org. Mass Spectrom.* **1991**, *26*, 903-907.; Lapko, V.N., Smith, D.L. and Smith, J.B. *Mass Spectrom.* **2000**, *35*, 572-575.], provided that the R_2 substituent used for the initial cysteine alkylation enhances sulfonium ion stability (i.e., the charge is stable) (step (ii) of Scheme 2). Importantly, conditions could be chosen so that the initial S-alkyl cysteine derivative is not stable to sulfonium ion formation, allowing specific derivatization of methionine containing peptides in cases where cysteines have been previously reduced and S-alkylated to enable efficient proteolysis during sample preparation. Neutral loss mode scan mode CID tandem mass spectrometry (MS/MS) scans, or post data acquisition neutral loss product ion detection software following conventional CID MS/MS, can be used to selectively identify methionine and cysteine containing peptides via the characteristic loss of CH_3SR_1 from methionine and R_1SR_2 from cysteine, with formation of $[M+(n+1)H-CH_3SR_1]^{(n+1)+}$ and $[M+(n+1)H-R_1SR_2]^{(n+1)+}$ product ions, respectively. Methionine and cysteine containing peptides can therefore be identified individually, through the use of an R_2 group that does not correspond to $-CH_3$, (i.e., the side chain alkyl group of methionine) or simultaneously, via initial alkylation of cysteine where the R_2 group corresponds to $-CH_3$.



Scheme 1



Scheme 2

In order for this derivatization strategy to be successful, both the solution phase and gas phase chemistries of the resultant sulfonium ions must be considered. These solution and gas phase chemistries, as well as characterization of the fragmentation behaviour of the fixed-charge sulfonium ion derivatives of methionine containing peptides have been examined using a series of model peptides designed to resemble those resulting from tryptic digestion, i.e., the class of peptides most commonly employed for protein identification by tandem mass spectrometry. The amino acid sequences of the model peptides ((GAILX$_1$GAILX$_2$), where X$_1$ is either alanine or methionine, and X$_2$ is either alanine, lysine or arginine), were chosen to allow comprehensive examination of the effects of charge state and amino acid composition on the observed fragmentation behaviour.

Solution phase requirements for sulfonium ion formation:

Reactions are easy to perform using readily available reagents. Many of the potentially useful reagents are commercially available. Other potentially useful reagents may be synthesized by simple methods described in the literature. Importantly, the ability to incorporate a suitable isotopic label into the selected derivative is an important consideration in allowing subsequent quantitation of differential protein expression using the same approach. Thus, isotopically labelled derivatives of these reagents should be commercially available or readily synthesized. For example, isotopically labeled d_5-bromoacetophenone has been readily prepared here from d_5-acteophenone using the brominating reagent, poly(4-vinylpyridinium tribromide), in a one-step process (see Materials and Methods). Reactions should be fast, proceed to completion and the resultant sulfonium ions must be chemically and thermally stable in solution [Stirling, M.J. and Patai, S. *The Chemistry of the Sulfonium Group*, Wiley: New York, **1981**.; Stirling C.J.M. *Sulfonium Salts in Organic Chemistry of Sulfur* Oae, S. Ed. Plenum Press NY **1977**, Chapter 9, pp 473-525.; Capozzi, G, and Modena, G. *Stud. Org. Chem.* **1985**, *19*, 246-298.]. Using the model peptide GAILMGAILK, a range of potential alkylating reagents for methionine side chain fixed-charge sulfonium ion formation, iodomethane, iodoethane, iodobenzene, iodomethylbenzene, iodoacetic acid, iodoacetamide and bromoacetophenone have been evaluated. The peptide was reacted for 4,

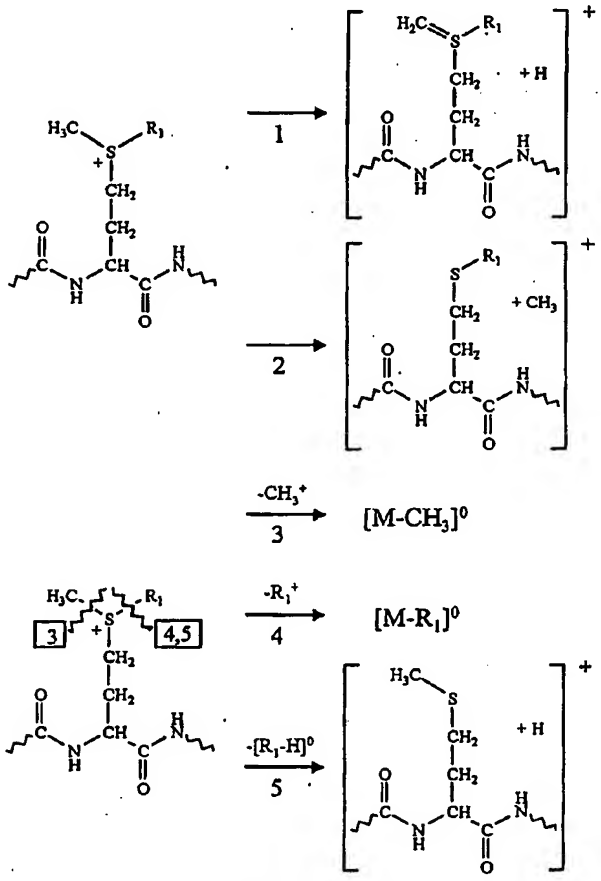
8, 16, and 32 hours with a 100 fold molar excess of each alkylating reagent then analysed by mass spectrometry to determine the extent of reaction. After 16 hours, only the acetophenone sulfonium ion derivative had reacted to completion [March J. "Advanced Organic Chemistry", 4th Ed., Wiley, New York, **1992**, pp 343.; Halvorsen, S. *J. Chem. Soc. Chem Comm*, **1978**, 327.; Yoh, L. *Tetrahedron Lett*, **1988**, *29*, 4431]. The ethyl derivative was found to have the least reactivity and stability in solution (only 1% reaction after 16 hours). All other reagents ranged in reactivity between these two extremes. The acetophenone derived sulfonium ion of the peptide was found to be stable in solution for several weeks without significant degradation. As free SH groups of cysteine residues have been shown to effect the reversal of the alkylation of methionine residues [Scheitjter, A. and Aviram, I. *FEBS Lett* **1972**, *21*, 293-296.], and transfer of a methyl group from a sulfonium salt to a free sulfhydryl acceptor is a well known possible side reaction [Toennies, G. and Kolb, J.J. *J. Am. Chem. Soc.*, **1945**, *67*, 1141-1144.; Naider, F. and Bohak, Z. *Biochemistry* **1972**, *11*, 3208-3211.; Cantoni, G.L. *Comp. Biochem.* **1960**, *1*, 181.], the commonly employed step involving S-alkylation of cysteines prior to proteolysis may be a useful but necessary reaction.

Gas phase requirements for sulfonium ion fragmentation

All sulfonium ions examined, including those with poor reactivity in solution, were found to be stable in the gas-phase so that they could be mass selected and manipulated for MS/MS experiments. All ions were found to give a unique fingerprint by fragmenting via the neutral loss of CH_3SR_1 (see Scheme 1). The loss of CH_3SR^1 from fixed-charge derivatives has been observed previously in small model systems [Reid, G.E., Simpson, R.J. and O'Hair, R.A.J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 1047-1060.; O'Hair, R.A.J. and Reid, G.E. *Eur. Mass Spectrom.* **1999**, *5*, 325–334.], peptides [Foti, S., Saletti, R. and Marletta, D. *Org. Mass Spectrom.* **1991**, *26*, 903-907.] and proteins [Lapko, V.N., Smith, D.L. and Smith, J.B. *Mass Spectrom.* **2000**, *35*, 572-575.]. Note that these gas phase reaction have solution phase analogies as discussed above. Side reactions such as charge migration via intramolecular proton transfer, alkyl group transfer, as well as breaking of bonds to give stable alkyl cations or alkenes must be avoided [O'Hair, R.A.J., Freitas, M.A., Gronert, S., Schmidt, J.A.R. and Williams, T.D. *J. Org. Chem.*, **1995**, *60*, 1990-1998.; Mudd, S.H. *et.*

al. Biochem, **1966**, *5*, 1653.; Deakyne, C.A. *et. al. J. Mol. Struct.* **1999**, 485/486, 33-41.; Markham, G.D. and Bock, C.W. *J Phys Chem*, **1993**, *97*, 5562-5569.; Katritzky, A.R. *et. al. Int J Mass Spectrom*, **1997**, *165/166*, 577-583.; Buckley, N. *et. al. J. Org. Chem.* **1996**, *61*, 2753-62.; Mestdagh, H. *et. al. Org. Mass Spectrom.* **1986**, *21*, 321-327.; Mestdagh, H. *et. al. Org. Mass Spectrom.* **1988**, *23*, 246-251.]. Proton transfer (Scheme 3, pathway 1) would give an sulfur ylide. Although the acidity of the $(CH_3)_3S^+$ ion (or the proton affinity of the conjugate base $(CH_3)_2S(CH_2)$) is unknown, sulfur ylides may be formed in solution by deprotonating sulfonium ions with bases such as hydroxide ion (HO-) or alkyl lithium reagents [Trost, B.M. and Melvin, L.S. "Sulfur Ylides: Emerging Synthetic Intermediates". Academic Press: NY **1975**.] Alkyl transfer (Scheme 3, pathway 2) is not likely to be a problem since intramolecular S_N2 rarely occur due to high energy transition states (co-linear geometry) [O'Hair, R.A.J., Freitas, M.A., Gronert, S., Schmidt, J.A.R. and Williams, T.D. *J. Org. Chem.*, **1995**, *60*, 1990-1998.]. The solution phase methyl cation affinities (MCAs) of some biological sulfur systems have been studied [Mudd, W.A., Klee P.D. *Biochem*, **1966**, *5*, 1653.]. Unfortunately, gas phase MCAs are limited to experimental and theoretical estimates for dimethylsulfide [Deakyne, C. A.; Knuth, D. M.; Meot-Ner, M.; Breneman, C. M.; Liebman, J. F. *J. Mol. Struct.* **1999**, *485/486*, 33-41] and theoretical estimates of some other sulfides [Markham, G.D. and Bock, C.W. *J Phys Chem*, **1993**, *97*, 5562-5569.]. The bond strengths of other breaking alkyl groups appear to be unknown in the both the condensed phase and gas phase. There have however, been several studies on the fragmentation reactions of sulfonium ions as studied by mass spectrometry [Katritzky, AR Shipkova, PA Watson, CH Eyler, JR. and Kevill DN. *Int J Mass Spectrom*, **1997**, *165/166*, 577-583.; Buckley, N; Maltby, D; Burlingame, A L.; Oppenheimer, N J. *J. Org. Chem.* 1996, 61, 2753-62; (d) Mestdagh, H; Morin, N; Rolando, C. *Org. Mass Spectrom.* **1986**, *21*, 321-327.; Mestdagh, H; Morin, N; Rolando, C. *Org. Mass Spectrom.* **1988**, *23*, 246-51.]. Scheme 3 (pathway 3) is highly unlikely given that the least stable carbocation is formed (CH_3^+). Problems associated with Scheme 3 (pathways 4 and 5) can be avoided by choosing R_1 groups which do not yield stable ions (e.g. avoid benzyl groups etc) and which do not form an alkene (e.g. avoid ethyl or substituted ethyl groups and higher homologues), respectively. Differentiation between the fragmentation reactions of sulfonium ions of methionine and S-alkyl cysteine residues (i.e., loss of CH_3SR_1 from

methionine (Scheme 1) versus loss of R_1SR_2 from cysteine (Scheme 2) is straightforward. This can be achieved here by using an alkyl group R_2 which is NOT a methyl group (i.e. $R_2 \neq CH_3$).



Scheme 3

General structure of the sufonium ion reagents suitable for use in the work described here.

Of the reagents examined here to date, the α-carbonyl containing alkylating reagents, Iodoacetic acid, iodoacetamide and bromoacetophenone are those that exhibit favourable solution and gas phase reaction properties. Of these, acetophenone allows ready incorporation of a suitable isotopic label (for

example, via synthesis of d_5-bromoacetophenone), to enable subsequent quantitation, and has therefore been the reagent used from here on. However, any reagent having the general structure XR, where X is any suitable leaving group consisting of, for example, halides, sulfonic esters, perchlorate esters or chlorosulfonates [March J. "Advanced Organic Chemistry", 4th Ed., Wiley, New York, **1992**, pp 352-357], and R is any substrate with a carbon adjacent to the leaving group, such as alkyl, allyl or α-carbonyl groups, with the exception of those indicated above, and where an isotopic or structural label can be incorporated to allow its use for differential quantitation, would be of interest.

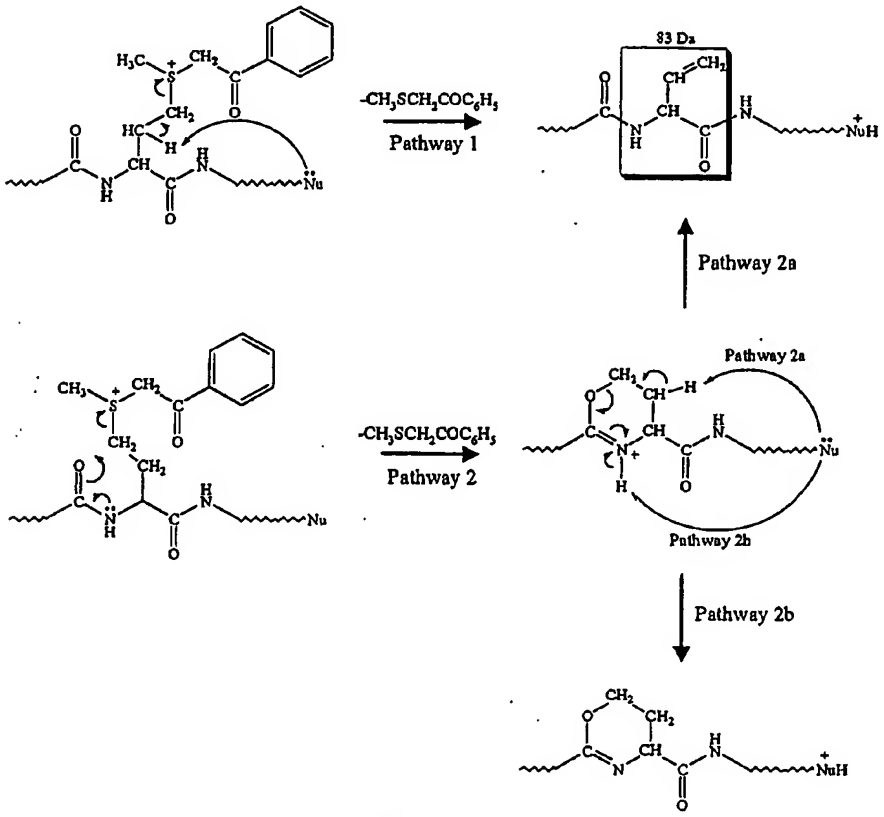
Examination of the fragmentation behaviour of fixed-charge sulfonium ion derivatives of methionine:

CID MS/MS and MS^3 in a quadrupole ion trap mass spectrometer.

The product ion spectra obtained by CID MS/MS of the singly and doubly charged ions of the unmodified and the acetophenone side chain derivatized sulfonium ion methionine containing peptides of GAILMGAILX, where X is either alanine, lysine or arginine, and the triply charged lysine and arginine containing ions of the acetophenone side chain derivatized sulfonium ion methionine containing peptides, in a quadrupole ion trap, are shown in Figures 1 to 7. MS/MS product ion spectra resulting from fragmentation of the singly and doubly protonated unmodified methionine containing peptide ions are shown in Figures 1A to 6A, while the spectra for the singly, doubly and triply charged acetophenone side chain derivatized sulfonium methionine containing peptide ions are shown in the insets to Figures 1B to 6B and in the insets to Figure 7A and B. The singly and doubly charged forms of the unmodified peptide ions, ionized by the addition of protons only, were all observed to form product ions resulting primarily from dissociations along the peptide backbone, with b- and y-type ions dominating. In contrast, the acetophenone side chain sulfonium methionine containing peptide ion derivatives each contain a single fixed-charge resulting from the sulfonium ion, with 0, 1 or 2 protons making up the balance for the singly, doubly or triply charged ions, respectively. For these ions, in each case the overwhelmingly dominant fragmentation pathway following CID MS/MS of the isolated precursor ion corresponded to selective cleavage of the α-CH_2-S bond at the site of the fixed-charge on the methionine

side chain, to yield the neutral side chain loss of S-methyl acetophenone ($CH_3SCH_2COC_6H_5$) (labelled as $-CH_3S(AP)$). Therefore, it is important to note that product ions formed by neutral loss from the side chain have the same charge state (and number of ionizing protons) as that of the precursor ion. The triply charged ion examples were the only partial exceptions, where some charged side chain loss of protonated S-methyl acetophenone ($CH_3SCH_2COC_6H_5+H^+$), labeled as ($-CH_3S(AP)+H^+$) in the insets to Figures 7A and B, (with the corresponding protonated S-methyl acetophenone ($CH_3S(AP)+H^+$) ion also labeled), was also observed. It is expected that the charged side chain loss from the triply charged precursor ions occurs by transfer of a proton from the neutral loss product ion following dissociation of the precursor ions covalent α-CH_2-S bond, but before dissociation of the resultant ion-molecule complex to the individual products, due to columbic repulsion.

A potential mechanism, with two possible pathways for the neutral loss of $CH_3SCH_2COC_6H_5$ from the fixed-charge derivatives is shown in Scheme 4.



Scheme 4

Pathway 1 of Scheme 4 involves direct proton transfer by an intramolecular nucleophile (Nu), such as the basic side chains of arginine or lysine, the N-terminal amino group or backbone amide carbonyl groups, with elimination of the neutral to yield a novel amino acid derivative, 3-amino-1-butenoic acid (vinyl glycine), with a residue mass of 83 Da. Alternatively, the fragmentation may be directed by a nucleophilic attack mechanism, whereby an adjacent amide carbonyl attacks the side chain to facilitate the neutral loss, yielding a cyclic product (shown in Pathway 2 of Scheme 4 as the 6 membered cyclic product formed from nucleophilic attack by the amide carbonyl on the N-terminal side of the modified methionine residue). This mechanism is consistent with the growing recognition that nucleophilic attack processes are the major processes involved in the fragmentation of protonated peptide ions [O'Hair,

R.A.J. *J. Mass Spectrom.* **2000**, *35*, 1377-1381. Schlosser, A. and Lehmann, W.D *J. Mass Spectrom.* **2000**, *35*, 1382-1390]. Intramolecular transfer of the acidic proton present in either the precursor ion (Pathway 1), or the product ion formed following methionine side chain cleavage (Pathway 2A or 2B) would result in "mobilization" of this proton, thereby allowing it to be involved in subsequent fragmentation reactions according to the mobile proton model developed for rationalizing the fragmentation of protonated peptide ions [Wysocki, V.H., Tsaprailis, G., Smith, L.L. and Breci, L.A. *J. Mass Spectrom.* **2000**, *35*, 1399-1406.]. Note that if the reaction proceeds via pathway 2 of Scheme 4, the amide bond incorporated into the cyclic product would not be amenable to subsequent cleavage. However, ring opening of the cyclic product via intramolecular transfer (Pathway 2A) would yield the acyclic amino acid derivative, thereby allowing subsequent fragmentation of the product ion to occur on both N- and C-terminal sides of the newly formed amino acid derivative.

Given that the sulfonium ion derivatized peptides fragment almost exclusively via the neutral loss of $CH_3SCH_2COC_6H_5$ to form a charged product ion, the neutral loss product ion for each peptide ion was mass selected and then subjected to a further stage of tandem mass spectrometry (MS^3) to generate additional structural information to obtain evidence for the preferred fragmentation pathway and also to allow its identification by sequence analysis. The spectra obtained following CID MS^3 of each of the neutral loss product ions discussed above are shown in Figures 1B to 6B and in Figure 7A and B. Similar to that of the singly and doubly protonated unmodified peptide ions, MS^3 of the $[M+nH-CH_3SCH_2C_6H_5]^{n+}$ product ions formed by neutral loss from the fixed-charge derivatives resulted in the formation of products resulting primarily from dissociations along the peptide backbone, with b- and y-type ions dominating. A direct comparison of the MS/MS product ion spectra of the unmodified peptides and the MS^3 spectra of the $[M+nH-CH_3SCH_2C_6H_5]^{n+}$ product ions reveals a number of interesting points;

(i) in most cases, cleavage of the amide bonds on either side of the modified vinyl alanine residue formed by loss of the sulfonium ion side chain was observed following MS^3, indicating that the fragmenting species did contain an acyclic vinyl glycine residue formed either by pathway 1 of Scheme 4 or by ring opening of the cyclic product formed by pathway 2 of Scheme 4;

(ii) for the singly charged methionine side chain fixed-charge peptide ions (Figures 1B, 3B and 5B), more extensive "sequence ion" fragmentation was observed following MS^3 of the $[M+nH-CH_3SCH_2C_6H_5]^{n+}$ product ions, with less "non-sequence" ion cleavages such as loss of water or ammonia and formation of a-type product ions, compared to that obtained by MS/MS of the unmodified methionine containing peptides. This observation may be readily explained by the mobile proton model for peptide fragmentation [Wysocki, V.H., Tsaprailis, G., Smith, L.L. and Breci, L.A. *J. Mass Spectrom.* **2000**, *35*, 1399-1406.]. In the case of the singly charged sulfonium ion peptide derivatives, the acidic proton formed upon loss of the side chain can be readily mobilized onto the peptide backbone by the direct proton transfer mechanism for cleavage in pathway 1 of Scheme 4, or by ring opening of the cyclic product ion shown in pathway 2A of Scheme 4, allowing subsequent fragmentation to take place at sites along the backbone, whereas, in the case of the singly protonated ions formed directly by electrospray, where the ionising proton is initially located on a basic side chain such as arginine or lysine, significant energy to overcome the barriers to proton transfer must be initially supplied in order for subsequent fragmentation of the backbone to occur. As such, other processes involving the loss of small molecules from the precursor and product ions such as water and ammonia, and formation of a-type product ions can therefore successfully compete with backbone cleavage. For the other peptides, the product ions formed by MS^3 from the doubly protonated lysine and arginine containing methionine side chain fixed-charge $[M+nH-CH_3SCH_2C_6H_5]^{n+}$ MS/MS product ions (Figures 4B and 6B) were similar to those obtained from MS/MS of the unmodified doubly protonated peptide ions. However, MS^3 of the doubly protonated product ion formed from dissociation of the acetophenone modified GAILMGAILA peptide (Figure 2B) yielded less extensive products than those formed by MS/MS of the unmodified peptide, with b_9 and b_9^{2+} ions dominating. This suggests that in this case, the proton initially present in the precursor and the acidic proton formed by loss of the side chain from the doubly charged sulfonium ion derivative are not as "mobile" as those of the doubly protonated peptide where one proton is expected to reside on the amino terminal and one along the backbone; again consistent with an initial cyclic product formed from the fixed-charge

derivative. The triply protonated lysine and arginine containing methionine side chain fixed-charge $[M+nH-CH_3SCH_2C_6H_5]^{n+}$ MS/MS product ions (Figures 7A and 7B, respectively) were found to yield mainly y_8^{2+} product ions, with other y-type ions also evident at low abundance;

(iii) the collision energy required for MS^3 of the $[M+nH-CH_3S(AP)]^{n+}$ product ions was consistently higher than that required for MS/MS of the unmodified peptide $[M+nH]^{n+}$ precursor ions for each of the charge states. This suggests that the fragmentation of the precursor ions formed by neutral loss have higher activation barriers compared to the precursors formed directly by electrospray ionization, and is again suggestive of the initial formation of the cyclic product ion in pathway 2 in Scheme 4 from the fixed-charge derivatives, with a necessity for ring opening prior to subsequent further dissociation.

Energy resolved CID MS/MS and in a quadrupole-time-of-flight mass spectrometer.

In order to determine the relative energetics of the fragmentation processes observed for the fixed-charge derivatives compared to the unmodified methionine containing peptides as well as those containing an alanine residue at the equivalent position to methionine (alanine has the closest structure of the common acids to the vinyl alanine residue formed by neutral loss from the fixed-charge derivatives and was therefore considered to be a better derivative than methionine for direct comparison of the relative energies required for fragmentation. These peptides were subsequently found to exhibit very similar product ion types and abundances compared to their methionine containing counterparts (data not shown)), and to obtain evidence for the structure of the resultant product ions, the fragmentation reactions of the sulfonium derivatives described above have been further examined by energy resolved CID in a quadrupole-time-of-flight (Q-TOF) mass spectrometer.

The energy resolved CID breakdown and appearance curves for the doubly charged sufonium ion derivative of GAILMGAILK is shown in Figure 8. The breakdown curve of the precursor $[M(AP)+H]^{2+}$ ion, the appearance/breakdown curve of the initial neutral loss product ion ($-CH_3S(AP)$), as well as the appearance curves of the individual product ions and that of the summed product ion abundances (sum of product ions) are indicated. It can be

seen that there is essentially no overlap between the disappearance of the $[M(AP)+H]^{2+}$ ion and the appearance of the individual product ions, indicating that these ions are not formed directly from the initial precursor. Rather, these ions are formed from the initial neutral loss product ion ($-CH_3S(AP)$). Representative product ion spectra taken under collision energies sufficient to obtain 70-90% reduction of the precursor ion abundance (12eV) and 70-90% reduction of the initial neutral loss product ion abundance (22eV) are shown in Figure 9A and B, respectively. The observed product ion spectra under these "low" and "high" energy quadrupole CID conditions, respectively, were essentially identical to those observed in the quadrupole ion trap mass spectrometry MS/MS and MS^3 experiments, indicating that the time frame (millisecond versus microsecond time scales for the ion trap and quadrupole experiments, respectively), and mode of ion activation had no appreciable effect on the fragmentation process. Indeed, all of the peptides examined under these "low" and "high" energy quadrupole CID conditions exhibited very similar product ion spectra compared to the equivalent ion trap derived MS/MS and MS^3 spectra (data not shown). Thus, the directed fragmentation of the fixed-charge derivatives gives the ability to use low energy conditions for formation of the initial neutral loss product ion and then to obtain a "pseudo MS^3" spectrum under "high" energy CID conditions to obtain further structural information.

The energy resolved breakdown/appearance curves for the formation of the initial neutral loss product ions from each of the singly and doubly charged precursors of the sulfonium ion derivatised methionine containing peptides (labeled as (A)-$CH_3S(AP)$, (K)-$CH_3S(AP)$ and (R)-$CH_3S(AP)$), as well as the appearance curves for their summed product ion abundances, are shown in Figures 10 and 11, respectively. The energy resolved breakdown/appearance curves for the formation of the initial neutral loss product ions from the triply charged precursors of the sulfonium ion derivatised methionine containing peptides (labeled as (K)-$CH_3S(AP)$ and (R)-$CH_3S(AP)$), and the appearance curves for their summed product ion abundances, are shown in Figure 12. For comparison, the appearance curves for the formation of the summed product ion abundances of the singly and doubly protonated unmodified methionine and alanine containing peptides are shown in Figure 13A and 13B, respectively.

A summary of the collision energy required to achieve 50% formation of product ions from the protonated unmodified peptides, and from the initial neutral loss product ions from the fixed-charge derivatives is given in Table 1.

These data can be used as a measure of the stabilities of the sulfonium ion derivative neutral loss product ions relative to their unmodified forms. The data indicates that while the collision energies required for dissociation of the alanine and unmodified methionine containing peptides is quite similar (generally within 0.5 to 1.5 V), the initial neutral loss product ions consistently required higher collision energies to achieve the same level of dissociation, giving further support for the formation of an initial cyclic product ion which is required to overcome a barrier to ring opening prior to subsequent dissociation (Scheme 4, pathway 2A).

Table 1. Collision Energy required to achieve 50% reduction in the abundance of the unmodified precursors and the initial neutral loss product ions from the fixed-charge sulfonium ion derivatives in a Q-TOF mass spectrometer.

Peptide Ion Charge State			
	+1	+2	+3
Peptide	Collision Energy (V)		
GAILAGAILA	21.7	8.1	NA
GAILMGAILA	22.7 (+1.0)[a]	8.6 (+0.5)[a]	NA
GAILM(AP)GAILA[b]	34.5 (+12.8)[a]	14.8 (+6.7)[a]	NA
GAILAGAILK	36.8	10.8	NA
GAILMGAILK	38.4 (+1.6)[a]	11.4 (+0.6)[a]	NA
GAILM(AP)GAILK[b]	43.0 (+6.2)[a]	21.5 (+10.7)[a]	11.6
GAILAGAILR	47.1	10.8	NA
GAILMGAILR	48.6 (+1.5)[a]	11.6 (+0.8)[a]	NA
GAILM(AP)GAILR[b]	53.7 (+6.6)[a]	22.1 (+11.3)[a]	11.7

[a] Difference between the collision energy required for 50% dissociation of the peptide of interest and its corresponding unmodified alanine containing peptide ion.

[b] (AP) = acetophenone side chain modification to methionine

As indicated above, an alternative to performing an additional stage of CID MS/MS, is the use of high resolution mass analyzers to obtain mass accuracies approaching 1 ppm (an "accurate mass tag") coupled with database searching, could be employed for subsequent identification of those peptides found to contain a fixed charge derivative. Previously, in cases where an

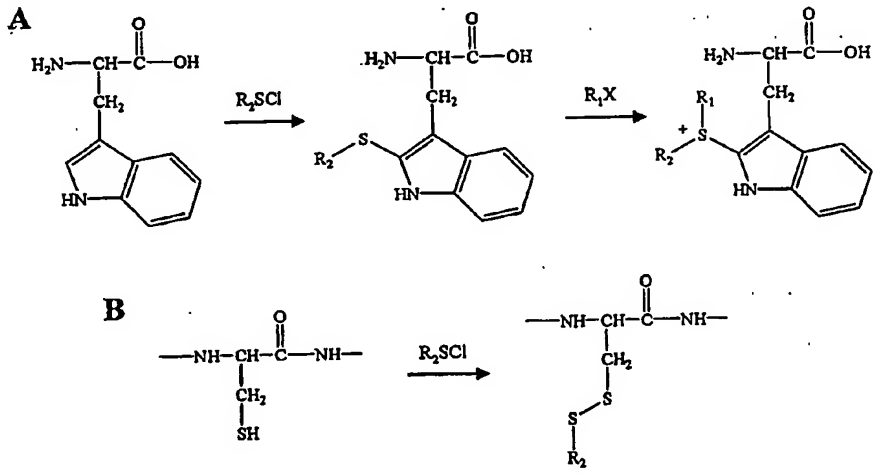
"accurate mass tag" has been obtained and the presence of a particular amino acid is known (for example the presence of a cysteine residue), the specificity of database searching algorithms can be improved such that unambiguous identification of the protein from which the peptide is derived has been achieved from this information alone [Conrads, T.P., Anderson, G.A., Veenstra, T.D., Pasa-Tolic, L. and Smith, R.D. *Anal. Chem.* **2000**, *72*, 3349-3354.; Goodlet, D.R., Bruce, J.E., Anderson, G.A., Rist, B., Pasa-Tolic, L., Fiehn, O., Smith, R.D. and Aebersold, R. *Anal. Chem.* **2000**, *72*, 1112-1118.].

Strategies for protein identification by CID MS/MS of peptides containing other amino acid fixed-charge derivatives.

Tryptophan

Modification of tryptophan by electrophilic aromatic substitution to yield a fixed-charge sulfonium ion derivative could be performed by initial reaction of both tryptophan and cysteine residues with a sulfenylhalide (R_2SCl) to yield derivatives with a thioether functional group in the 2 position of the indole side chain for tryptophan (Scheme 5A) and an unsymmetrical disulfide of cysteine (Scheme 5B), followed by the formation of the sulfonium ion derivative of tryptophan under conditions identical to those discussed above for methionine (Scheme 5A). The specificity of the sulfenylhalide reaction to tryptophan and cysteine amino acid residues has previously been examined extensively. [Scoffone, E., Fontana, A. and Rochhi, R. *Biochemistry.* **1968**, *7*, 971.]. The specificity of the reaction step involving the fixed-charge sulfonium ion derivatization of tryptophan and not cysteine is inherently imparted by the formation of a non-symmetrically bonded cysteine disulfide in the first step of the reaction, which will not be amenable to further reaction. Note that the same solution phase and gas phase criteria for sulfonium ion formation of tryptophan residues apply as discussed above for methionine and for S-alkyl cysteine. As sulfonium ion derivatives of the 2-indole S-alkyl derivatives of tryptophan will be formed under conditions identical to those discussed above for methionine and S-alkyl cysteine, both residues would be modified in this approach. However, as for the reactions of methionine and S-alkyl cysteine sulfonium ion derivatives discussed above, methionine and tryptophan containing peptides could be identified individually, through the use of an R_2 group that does not correspond

to $-CH_3$, (i.e., the side chain alkyl group of methionine) or simultaneously, via initial alkylation of tryptophan where the R_2 group corresponds to $-CH_3$. Likewise, 2-indole S-alkyl tryptophan and S-alkyl cysteine containing peptides could be identified individually, through the introduction of an R_2 group to the S-alkyl tryptophan derivative that does not match that of the R_2 group used for initial alkylation of cysteine, or simultaneously, via initial alkylation of tryptophan using the same R_2 group as that used for initial cysteine alkylation. Similar electrophilic aromatic substitution reactions could be used for the introduction of an S-alkyl group to the side chain of tyrosine residues, followed by subsequent formation of a sulfonium ion derivative.

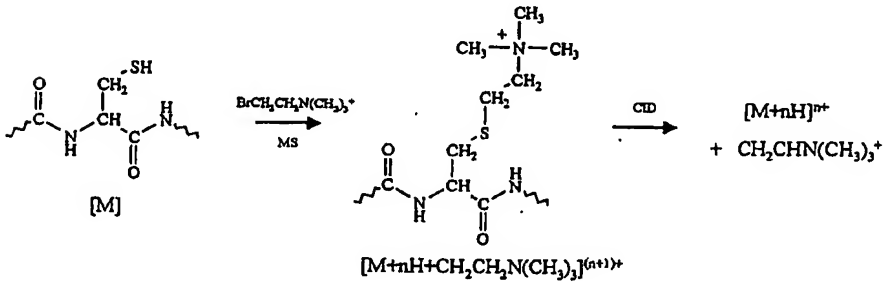


Scheme 5

Cysteine

In contrast to the fragmentation reactions discussed above for the fixed-charge sulfonium ions, where neutral losses were observed as the exclusive dissociation reaction products, the directed fragmentation of peptides derivatized on their side chains with fixed-charge quaternary alkylammonium or quaternary alkylphosphonium ion reagents should exclusively result in charged loss of the side chain, with formation of a dominant low mass-product ion (for example m/z 86 for the fragmentation of an S-ethyl, trimethylammonium ion derivative of cysteine shown in Scheme 3) as well the formation of its

complementary neutral peptide species (if the initial charge state of the precursor was one), or a charged peptide ion species with a charge state one lower than the precursor (if the initial charge state of the precursor was greater than one). The characteristic low mass product ion can be selectively detected using a parent ion scan mode MS/MS experiment. The fragmentation behaviour of tertiary alkyl fixed-charge derivatives of cysteine have not been examined previously. However, the fragmentation of cysteine-containing peptides alkylated with reagents containing basic functionalities, such as iodoacetamide, acrylamide and vinylpyridine, have been observed previously to give rise to some fragmentation of the side chain [Moritz, R.L. Eddes, J.S., Reid, G.E. and Simpson, R.J. *Electrophoresis.* **1995**, *17*, 907-917.]. It is expected that side chain cleavage could be further enhanced to become the sole fragmentation pathway observed upon introduction of a fixed-charge derivative. The same solution phase and gas phase requirements for quaternary alkylammonium and quaternary alkylphosphonium ion formation and fragmentation apply as discussed above for sulfonium ions. A fixed-charge derivative of cysteine (using (2-bromoethyl)trimethylammonium bromide ($BrCH_2CH_2(CH_3)_3NBr$) for introduction of the fixed charge to the side chain thiol of the cysteine residue), has been used previously to enable carboxypeptidase C-terminal peptide sequence analysis, with mass spectrometry used for detection of the reaction products [Bonetto, V., Bergman, A-C., Jornvall, H. and Sillard, R. *Anal.Chem.* **1997**, *69*, 1315-1319.].



Scheme 6

General structure of the tertiary alkyl fixed-charge ion reagents suitable for use in the work described here.

Any reagent having the general structure XR, where X is any suitable leaving group consisting of, for example, halides, sulfonic esters, perchlorate esters or chlososulfonates [March J. "Advanced Organic Chemistry", 4th Ed., Wiley, New York, **1992**, pp 352-357], and R is any substrate with a quaternary alkylammonium or quaternary alkylphosphonium ion adjacent to the leaving group, and where an isotopic or structural label can be incorporated to allow its use for differential quantitation, would be of interest.

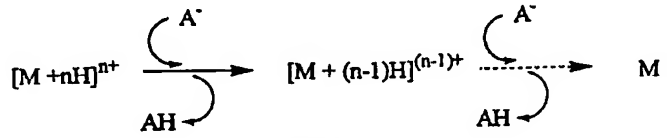
Other mass spectrometry instrumentation and ionization source considerations.

It is important to recognise that, as dissociation of sulfonium ion derivatized peptides predominantly results in the neutral loss of the side chain, with the resultant product ion having the same charge state (and number of ionising protons) as that of the precursor ion, the sulfonium ion MS/MS approach is applicable to both ESI (where multiply charged ions are usually observed) and MALDI (where predominantly singly charged ions are observed) ionization methods. In contrast, dissociation of quaternary alkylammonium or quaternary alkylphosphonium ion derivatives would yield charged side chain cleavage product ions, and therefore the peptide containing fragment would carry one less charge than that of the precursor ion. Thus, the tertiary alkylammonium or quaternary alkylphosphonium ion approach would be best carried out using ESI methods, where a charged product ion containing the peptide would be formed in addition to the charged side chain cleavage low mass product ion. One benefit of the fixed-charge derivatization approaches described here should be to yield an improved ionization response for either ionization methods, leading to greater sensitivity and less matrix suppression effects, as the ions are preformed and therefore do not have to "compete" for the available charge in either the charged droplets formed by ESI, or the matrix plume formed by MALDI.

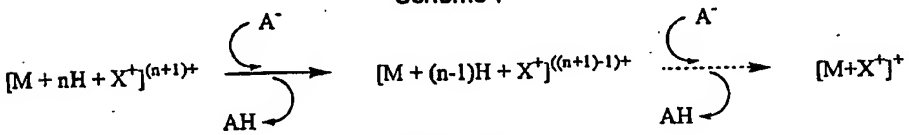
Identification of proteins by ion-ion reaction MS/MS of their fixed-charge derivative containing peptides.

If desired, any of the fixed-charge derivatives discussed above could also be examined by alternate tandem mass spectrometry methods. Although the term "tandem mass spectrometry" has traditionally been used to describe reaction events involving dissociation of the mass selected ion, many alternative reaction types that also fit the definition of a tandem mass spectrometric reaction sequence are commonly employed to obtain information about the structure and reactivity of ions in the gas-phase. Thus, an MS/MS (or MS^n) experiment may also include, for example, ion-molecule, ion/ion, and ion-mobility reaction events. It is therefore envisioned that other tandem mass spectrometry (MS/MS) methods in addition to those employing CID could also be used here for peptide identification, differential quantitation, as well as analysis of O-linked post translational modifications and cross-linking status. For example, the use of gas-phase ion-ion reactions to perform charge neutralization via proton transfer [Reid, G.E., Wells, J.M., Badman, E.R. and McLuckey S.A. *Int. J. Mass Spectrom.* **2002**, *In Press.*; Reid, G.E.; Shang, H.; Hogan, J.M.; Lee, G.U.; McLuckey, S.A.. *J. Am. Chem. Soc.* **2002**, *124*, 7353-7362.; McLuckey, S.A.; Reid, G.E.; Wells, J.M. *Anal. Chem.* **2002**, *74*, 336-346.; McLuckey, S.A.; Stephenson, J.L. *Mass Spectrom. Rev.* **1999**, *17*, 369-407.; Stephenson, J.L.; Mcluckey, S.A. *J. Mass Spectrom.* **1998**, *33*, 664-672.; Stephenson, J.L; McLuckey, S.A. *Anal. Chem.* **1998**, *70*, 3533-3544.; Stephenson, J.L.; Mcluckey, S.A. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 585-596.; McLuckey, Scott A.; Stephenson, James L.; Asano, Keiji G. *Anal. Chem.* **1998**, *70*, 1198-1202.; Stephenson, James L.; McLuckey, Scott A.. *Int. J. Mass Spectrom. Ion Proc.* **1997**, *165/166*, 419-431.; Stephenson, J.L.; McLuckey, S.A. *Anal. Chem.* **1997**, *69*, 3760-3766.; Stephenson, J.L..; McLuckey, S.A. *J. Am. Chem. Soc.* **1996**, *118*, 7390-7397.], could be employed to selectively "remove" all but the singly charged fixed-charge derivatives discussed above, thereby allowing their "enrichment" in the gas-phase. This approach therefore may be useful where fixed-charge derivatives are prepared so that they do not participate in fragmentation in any way but simply act as "spectators". For comparison, Schemes 7 and 8 indicate the general reaction for reaction between a singly charged anion and singly or multiply protonated, or fixed-

charge cationic precursors. Whereas complete neutralization of the protonated ions is expected upon sequential reaction with the anions (Scheme 7), precursors containing the fixed-charge ions would be reduced to their singly charged forms only (Scheme 8). As discussed above, those ions identified as containing fixed-charge derivatives via this approach could then be subjected to CID MS/MS to obtain structural information, or alternatively, via determination of an accurate mass tag, to enable their subsequent identification.



Scheme 7



Scheme 8

Differential quantitation of proteins by CID MS/MS of their fixed-charge derivative containing peptides.

Neutral loss scan mode CID MS/MS in a triple quadrupole mass spectrometer.

In a general strategy for differential quantitation using the fixed-charge ion approach as described here, a first sample is derivatized with either sulfonium or tertiary alkyl fixed-charge reagents containing naturally abundant (light) isotopes, are then mixed with a second sample derivatized with the same reagent containing isotopically enriched (heavy) isotopes, prior to introduction to the mass spectrometer. Then, using either neutral loss or parent ion mode CID MS/MS scans, whereby the mass spectrometer detects product ions offset by a given mass from the precursor ion (in the case of peptides containing sulfonium ion fixed-charge derivatives) or diagnostic low mass product ions (in the case of peptides containing tertiary alkylammonium ion fixed-charge derivatives), respectively, the abundance ratios of the product ions formed by neutral loss or parent ion MS/MS scans of the light isotope tag containing

peptide ions, compared to those containing heavy isotope tag can be determined and will be indicative of changes in the abundance levels of proteins between the two samples, thereby allowing their relative quantitation. The product ion representing the peptide can then be automatically selected for further structural interrogation by "high" energy CID as described above for the energy resolved CID experiments. Issues relating to overlap of labelled versus unlabelled peptides due to poor instrument resolution, or due to other peptides being present are therefore overcome, as the MS/MS product ion gives a "pure" precursor for quantitation and subsequent structural interrogation. The approach could also be readily combined with a "global" labelling (i.e., N-or C-terminal labelling) strategy, whereby the isotopic label is introduced at a position remote to the site of the fixed-charge so that a common neutral loss could be used to identify both light and heavy isotope containing derivatives.

The ESI mass spectrum of a nominal 1:1 mixture of the GAILMGAILR peptide derivatized with either d_0- or d_5- acetophenone and introduced by electrospray ionization to a triple quadrupole mass spectrometer is shown in Figure 14. From this mass spectrum, a ratio of 1.09:1 can be obtained for both the doubly (*m/z* 567.2 and 569.7) and triply (*m/z* 378.4 and 380.1) charged states of the peptide (Table 2). A 20 times expanded region of the spectrum around *m/z* 700-800 shows the level of chemical noise associated with the mass spectrum. In comparison, the neutral loss scan mode MS/MS spectra of the 1:1 d_0- and d_5- containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivatives of GAILMGAILR for the doubly charged d_0- and d_5- containing ions (neutral losses of 83 Da and 85.5 Da, respectively) and the triply charged d_0- and d_5- containing ions (neutral losses of 55.3 Da and 57 Da, respectively) are shown in Figure 15A and B, respectively. Again, the *m/z* 700-800 regions for each spectrum have been expanded 20 times to indicate the level of chemical noise associated with the MS/MS spectrum. While the observed ratios for the MS/MS neutral loss scan mode ion abundances were essentially identical to those observed for the MS ion abundances in Figure 14 (Table 2), the level of noise was reduced by at least two orders of magnitude compared to that observed in the mass spectrum in Figure 14. With the exception of electronic noise, characterised by single data point noise "spikes", the neutral loss MS/MS spectra showed a complete absence of non-specific chemical noise along the baseline.

Table 2 Differential Quantitation by MS versus neutral loss scan mode CID MS/MS methods.

	Peptide Ion Charge State					
	+2	+3		+2	+3	
Nominal Ratio	Observed Ratio (MS)			Observed Ratio (MS/MS)		
1:1	1.09	1.09	(1.09, 0%, +9%)[a]	1.08	1.10	(1.09, 1.8%, +9.0%)[a]
2:1	2.12	1.81	(1.965, 14.6%, -1.75%)[a]	2.05	1.93	(1.99, 5.85%, -0.05%)[a]
5:1	4.07	4.47	(4.27, 8.9%, -14.6%)[a]	4.50	4.63	(4.565, 2.8%, -8.7%)[a]

[a] The values in parentheses are the average ratio obtained for the two charge states subjected to analysis, the percent error between the two charge states, and the percent error of the average value compared to the nominal value.

For the neutral loss product ions formed from the sulfonium ion derivatized peptide precursors, a common product is formed from both "heavy" and "light" ions, therefore either product may be selected for further structural analysis by "high" energy CID. The "low" energy (17.5eV) CID MS/MS product ion spectra of the doubly charged d_0- containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion ($[M(AP)+H]^{2+}$) of GAILMGAILR obtained under conditions identical to the neutral loss mode MS/MS scan data described above is shown in Figure 16A. The MS/MS product ion spectrum obtained under "high" collision energy conditions (31V) is shown in Figure 16B. Similar data for the triply charged d_0- containing ion obtained using 13eV (the same collision energy was used for the neutral loss mode MS/MS scan data shown in Figure 15B), and 18eV collision energy conditions are shown in Figure 17A and B, respectively. The product ion spectra obtained under these conditions were essentially identical to those obtained in the ion trap or Q-TOF mass spectrometers, indicating again that the method of ion activation has little influence on the observed fragmentation behaviour.

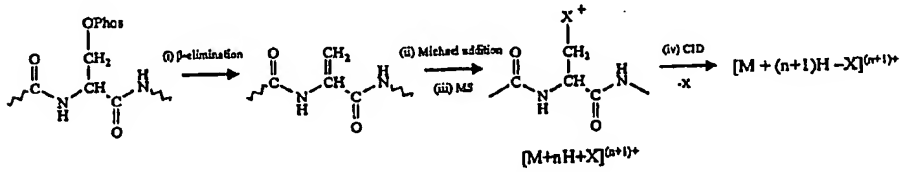
The mass spectra and neutral loss scan mode MS/MS spectra of nominal 2:1 and 5:1 mixtures of d_0- and d_5- containing methionine side chain fixed-charge acetophenone (AP) sulfonium ion derivative of GAILMGAILR are shown in Figures 18 and 19, and 20 and 21, respectively. The abundance ratios obtained for the doubly and triply charged neutral loss scan mode

MS/MS experiments were determined to be 2.05:1 and 1.93:1 for the nominal 2:1 mixture, and 4.50:1 and 4.63:1 for the nominal 5:1 mixture (see Table 2). The errors associated with these measurements, determined by calculating the percent errors observed between the two charge states, as well as by comparison of the average values with the expected nominal values were all found to be significantly smaller than those obtained from the mass spectra data (compare the observed ratio (MS) and observed ratio (MS/MS) values as well as the calculated errors in parenthesis for each ratio in Table 2). This reduction in error is presumably due to the reduction in chemical noise and improvement in sensitivity associated with the MS/MS measurements. It is unknown at this stage what role ion coalescence effects play in the ability to accurately measure the abundances of ions similar in mass as they are resolved by a mass spectrometer. If such effects do occur, it is possible that the ion abundances measured under MS conditions are systematically affected by the charge state of the ion being measured, therefore introducing greater disparity in the quantitative ratios than are actually present. These effects however, if relevant, are not expected to play a role when measuring product ion abundances resulting from the CID of isolated precursor ions, such as demonstrated here for the neutral loss scan mode experiments. One of the additional advantages of the MS/MS method described here over those involving identification by MS peak abundances alone is that both ions (i.e., light and heavy) are not required to be present in order for detection of one of the pairs to be achieved. Also, in these instances, the specific nature of the neutral loss from the parent ion indicates the origin of the ion (i.e., from the "light" or "heavy" sample).

Identification and differential quantitation of O-linked post translationally modified proteins by CID MS/MS or ion-ion reaction MS/MS of their peptides containing fixed-charge derivatives:

If combined with the β-elimination/Michael addition chemistry for forming mass spectrometry stable derivatives of serine and threonine O-linked post translationally modified amino acid containing peptides, the fixed-charge derivatization approach could be extended toward the identification and quantitation of O-linked post translational modification status in proteins. Following β-elimination of O-linker serine or threonine post translational

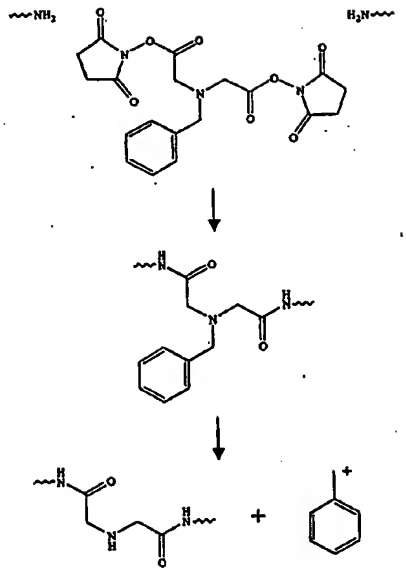
modifications, a one or two step Michael addition process could be used for fixed-charge introduction. Scheme 9 illustrates a one step process whereby the fixed-charge derivative (for example, a quaternary ammonium or phosphonium ion derivative) is introduced directly. Alternatively, in a two step process, a neutral side chain derivative could be introduced (e.g., an alkyl thiol), followed by subsequent derivatization to form a fixed charge sulfonium ion. Therefore, sulfonium, quaternary alkylammonium, or quaternary alkylphosphonium ion chemistries as outlined above, could also be used for selective detection of peptides containing the modified amino acid residue. A similar method has been described by Steen and Mann for the introduction of a dimethylamine-containing sulfenic acid derivative to β-eliminated phospho-serine and phospho-threonine residues, thereby allowing a characteristic protonated low mass fragment ion at *m/z* 122.06 to be detected by precursor ion scan mode MS/MS upon low energy CID (Steen, H. and Mann, M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 996-1003). However, this ion is not always observed as a dominant product and the formation of this ion can be competitive with other backbone fragmentation processes. Therefore, a mixture of product ions without and with the modified side chain can be present simultaneously, resulting in a complex product ion spectrum making it potentially difficult to interpret without prior knowledge of the expected peptide sequence. The fixed charge derivatization method described here however, would result in the exclusive loss of the modified side chain, allowing improved detection of these peptides at higher levels of sensitivity and with better control of the subsequent dissociation process for subsequent structural elucidation. When combined with the differential isotopic labelling strategy outlined above, the fixed charge derivatization method of the present invention would also allow differential quantitation of the O-linked post translational modification status.



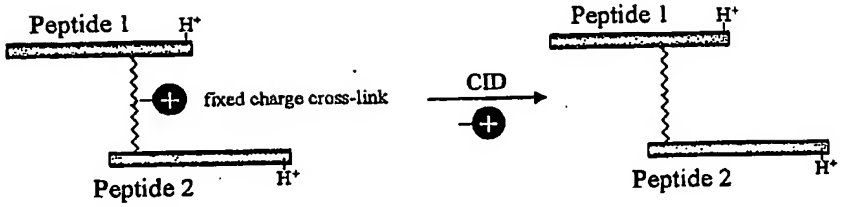
Scheme 9

Characterization of Protein-Protein Interactions by CID MS/MS or ion-ion reaction MS/MS Identification of cross-linked peptides containing fixed-charge derivatives.

The fixed-charge derivatization approach could also be extended toward the improved characterization of protein-protein interactions by incorporation of the fixed-charge derivative into a suitable cross-linking reagent prior to cross-linking reactions, or by deritivatization of a cross-link contained between two proteins or peptides after a cross-linking reaction which, upon CID MS/MS, fragments via the loss of a neutral (or charged) entity from the cross-link. While the incorporation of a labile MS/MS "tag" on a cross linker has been used by Back *ef al.* to identify cross linked peptides via the detection of a characteristic low mass product ion formed upon low energy CID [Back, J.W., Hartog, A.F., Dekker, H.L., Muijsers, A.O., de Koning, L.J. and de Jong, L. *J. Am. Soc. Mass Spectrom.* **2001**, 12, 222-227.] (Scheme 10), the benzyl ion thus formed is not the dominant cleavage product, and is seen as only a relatively low abundance product ion. In contrast, in a similar manner to that employed for peptide identification and quantitation using fixed-charge derivatives as outlined above, the method of the present invention could be employed here to direct the fragmentation of cross linked peptides toward exclusive cleavage of the fixed-charge, to yield either a characteristic neutral loss or low mass product ion (Scheme 11). The product ion containing the cross linked peptide may then be automatically selected for further structural interrogation by higher energy MS/MS or by MS^3. If quantitative comparison of cross linking between two different samples was required, an isotopic label could be also incorporated into the cross-link.



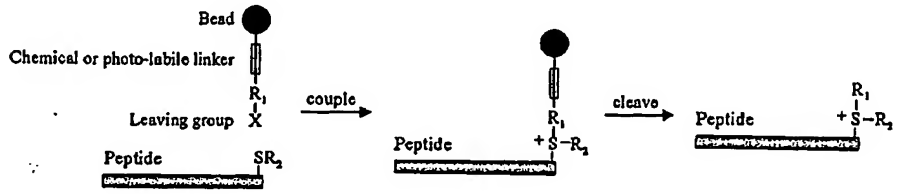
Scheme 10



Scheme 11

If desired, any of the examples discussed above containing fixed-charge derivatives could also be selectively pre-enriched prior to mass spectrometric analysis by known chromatographic methods [Tang, J.R. and Hartley, B.S. *Biochem. J.* **1967**, *102*, 593.; Degen, J. and Kyte, J. *Anal. Biochem.* **1978**, *89*, 529-539.; Kyte, J., Degen, J. and Harkins, R.N. *Methods Enzymology* **1983**, *91*, 367-377.; Gevaert, K., Van Damme, J., Goethals, M., Thomas, G.R., Hoorelbeke, B., Demol, H., Martens, L., Puype, M., Staes, A. and Vandekerckhove, J. *Mol. Cell. Proteomics.* **2002**, *In Press.*]. Alternatively the fixed-charge derivatives could be selectively pre-enriched by solid phase capture mehods, using fixed charge reagents covalently coupled to beads or insoluble polymers [Weinberger, S.R.; Viner, R.I. and Ho, P. *Electrophoresis.*

2002, *23*, 3182-3192.]. Chemical or photolytic cleavage [Zhou, H.; Ranish, J. A.; Watts, J. D.; Aebersold, R. *Nature Biotechnol.* **2002**, *20*, 512-515.; Qiu, Y; Sousa, E. A.; Hewick, R. M.; Wang, J. H. *Anal. Chem.* 2002, 74, 4969-4979.], would then be employed to release the fixed-charge derivative of the peptide (conceptually shown for a sulfonium ion fixed charge derivative in Scheme 12), thereby reducing the sample complexity of the mixture to be analysed.



Scheme 12

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The disclosures of the references cited herein are hereby incorporated herein by reference.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

Dated this eighteenth day of November 2002

Ludwig Institute for Cancer Research
Patent Attorneys for the Applicant:

F B RICE & CO

Figure 1

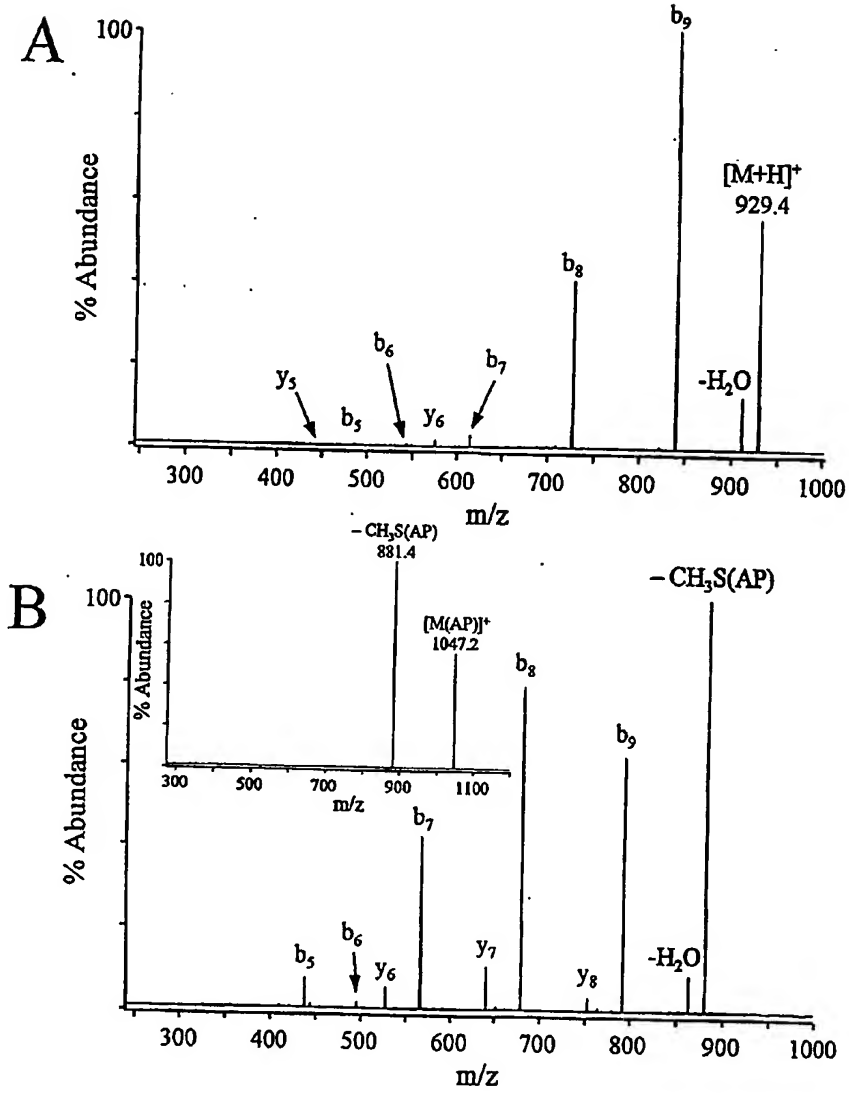
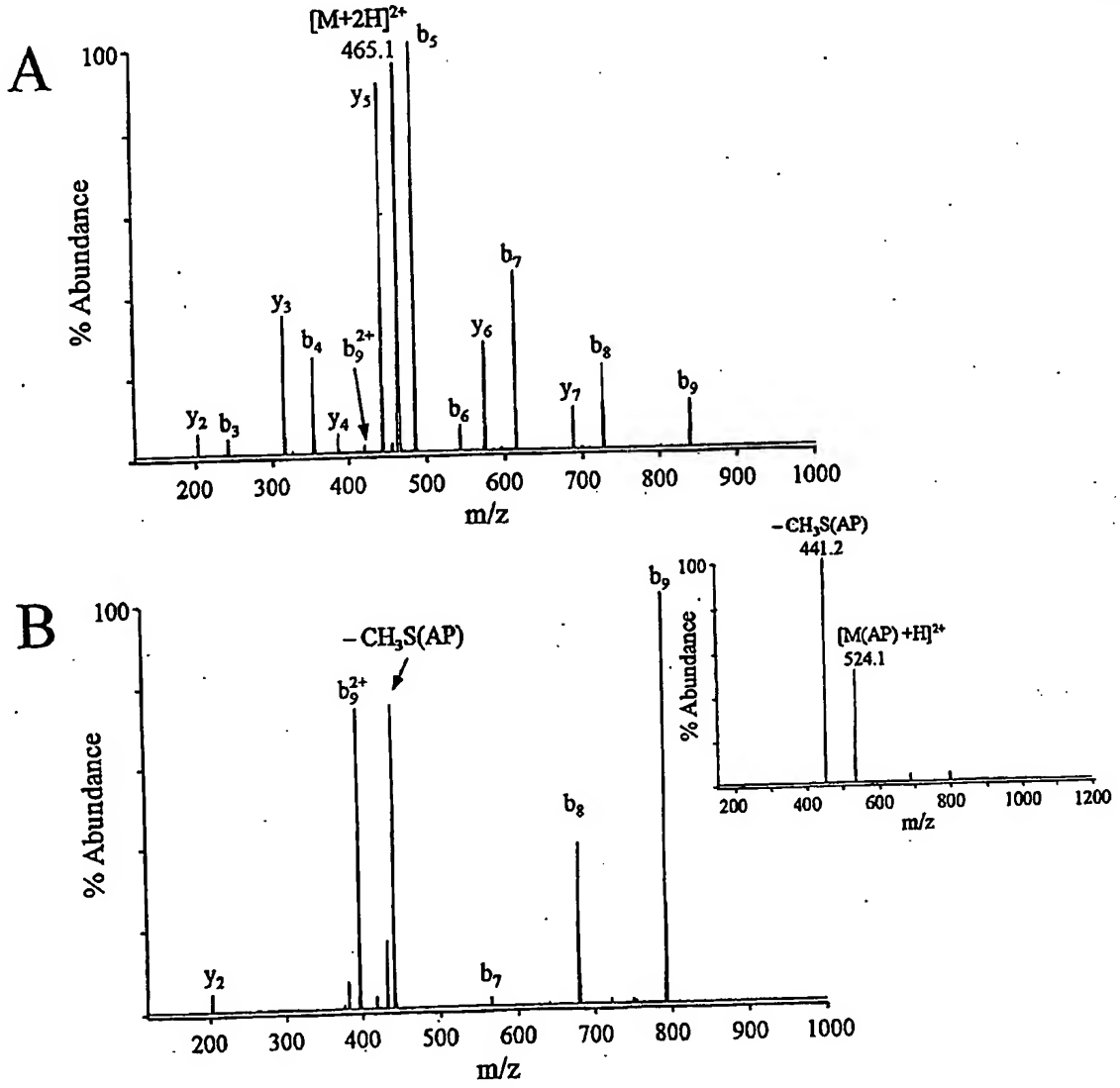

A
100
% Abundance
b9
[M+H]+
929.4
b8
b6
b7
y5
b5
y6
-H2O
300
400
500
600
700
800
900
1000
m/z
B
100
% Abundance
- CH3S(AP)
881.4
[M(AP)]+
1047.2
- CH3S(AP)
b8
b9
b7
b5
b6
y6
y7
y8
-H2O
300
500
700
900
1100
m/z
300
400
500
600
700
800
900
1000
m/z

Figure 2


A
100
% Abundance
[M+2H]2+
465.1
b5
y5
b7
y3
b4
b92+
y6
b8
y7
b9
y2
b3
y4
b6
200
300
400
500
600
700
800
900
1000
m/z
B
100
% Abundance
-CH3S(AP)
b92+
b9
b8
y2
b7
200
300
400
500
600
700
800
900
1000
m/z
-CH3S(AP)
441.2
[M(AP)+H]2+
524.1
100
% Abundance
200
400
600
800
1000
1200
m/z

2/21

Figure 3

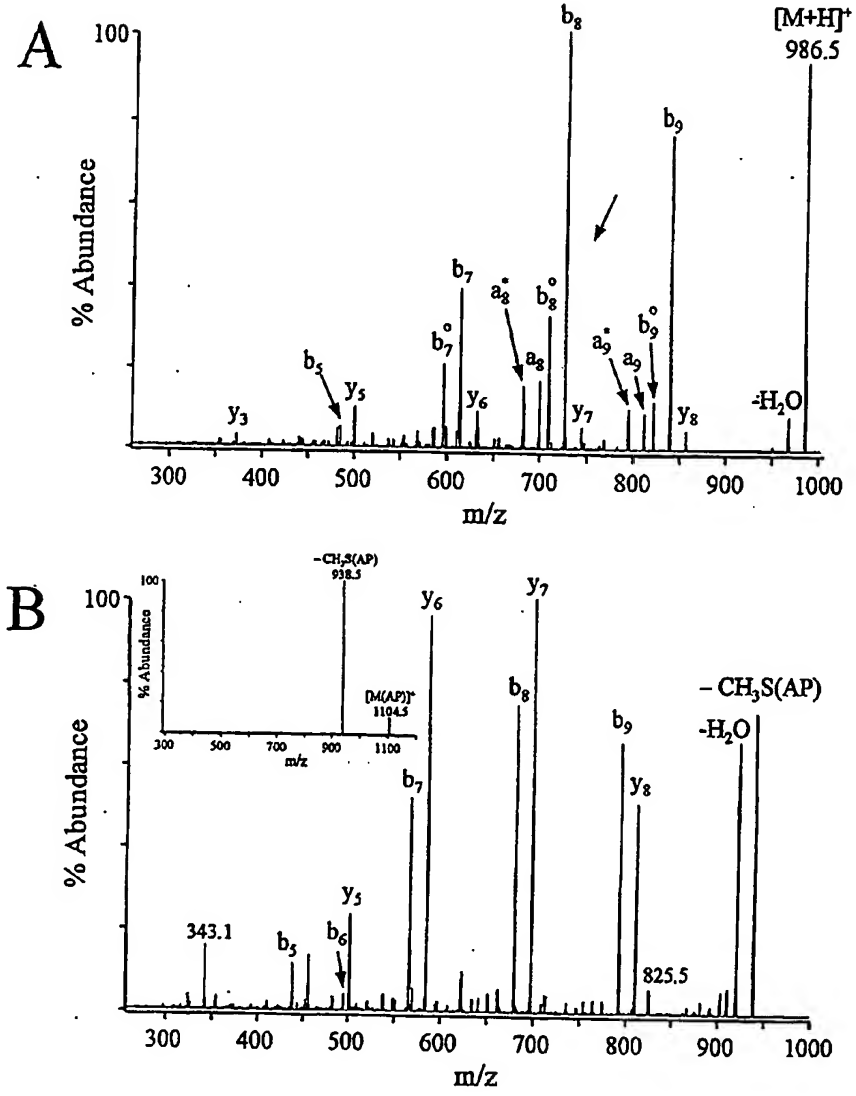
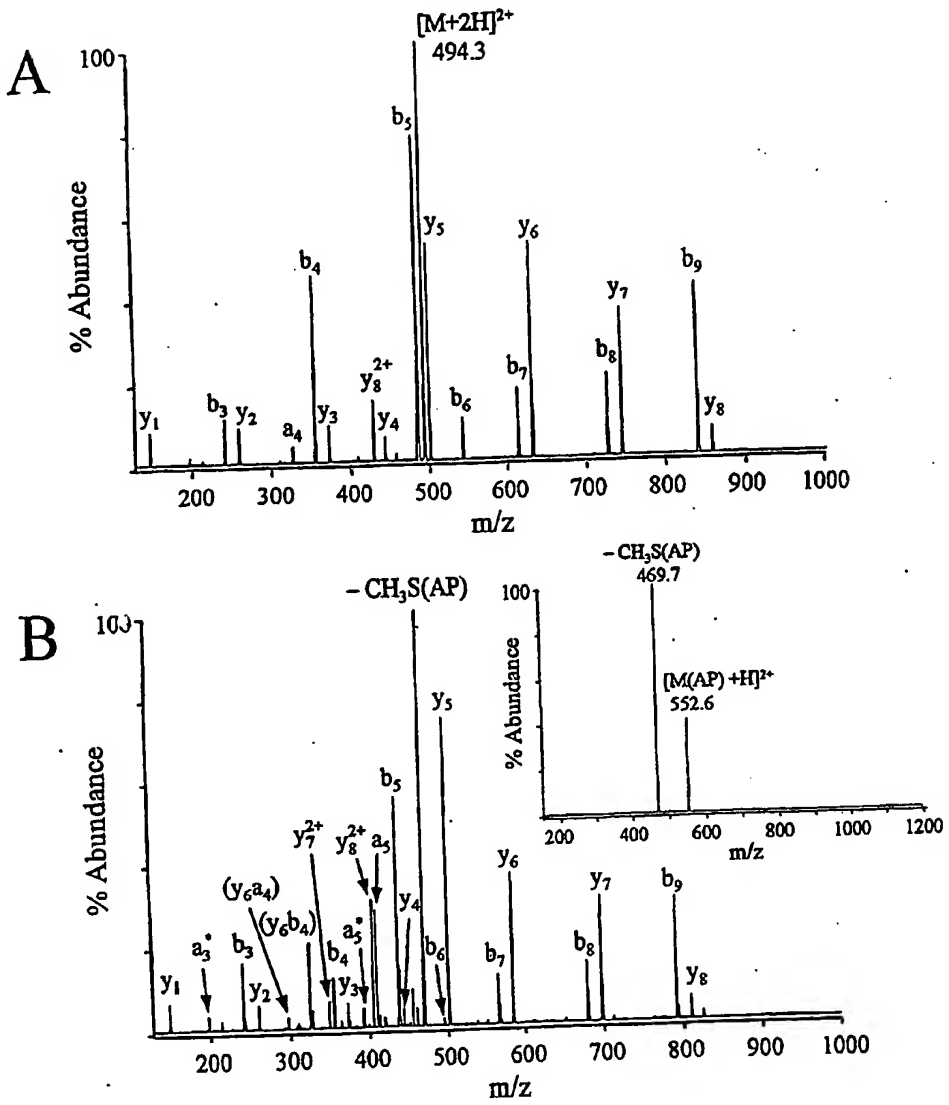

A
100
% Abundance
b8
[M+H]+
986.5
b9
b7
a8*
b8°
b7°
a8
b5
y5
y6
a9*
a9
b9°
y3
y7
y8
-H2O
300
400
500
600
700
800
900
1000
m/z
B
100
% Abundance
-CH3S(AP)
938.5
[M(AP)]+
1104.5
300
500
700
900
1100
m/z
y6
y7
b8
b9
- CH3S(AP)
-H2O
b7
y8
y5
343.1
b5
b6
825.5
300
400
500
600
700
800
900
1000
m/z

Figure 4


A
[M+2H]2+
494.3
100
% Abundance
b5
y5
y6
b4
b9
y7
b8
y8 2+
b7
b3 y2
y1
a4
y3
y4
b6
y8
200 300 400 500 600 700 800 900 1000
m/z
B
- CH3S(AP)
100
% Abundance
y5
b5
y7 2+
y8 2+
a5
y6
y7
b9
(y6a4)
(y6b4)
y4
a3*
b3
a5*
b4
b6
b7
b8
y8
y1
y2
y3
200 300 400 500 600 700 800 900 1000
m/z
- CH3S(AP)
469.7
[M(AP) +H]2+
552.6
100
% Abundance
200 400 600 800 1000 1200
m/z

4/21

Figure 5

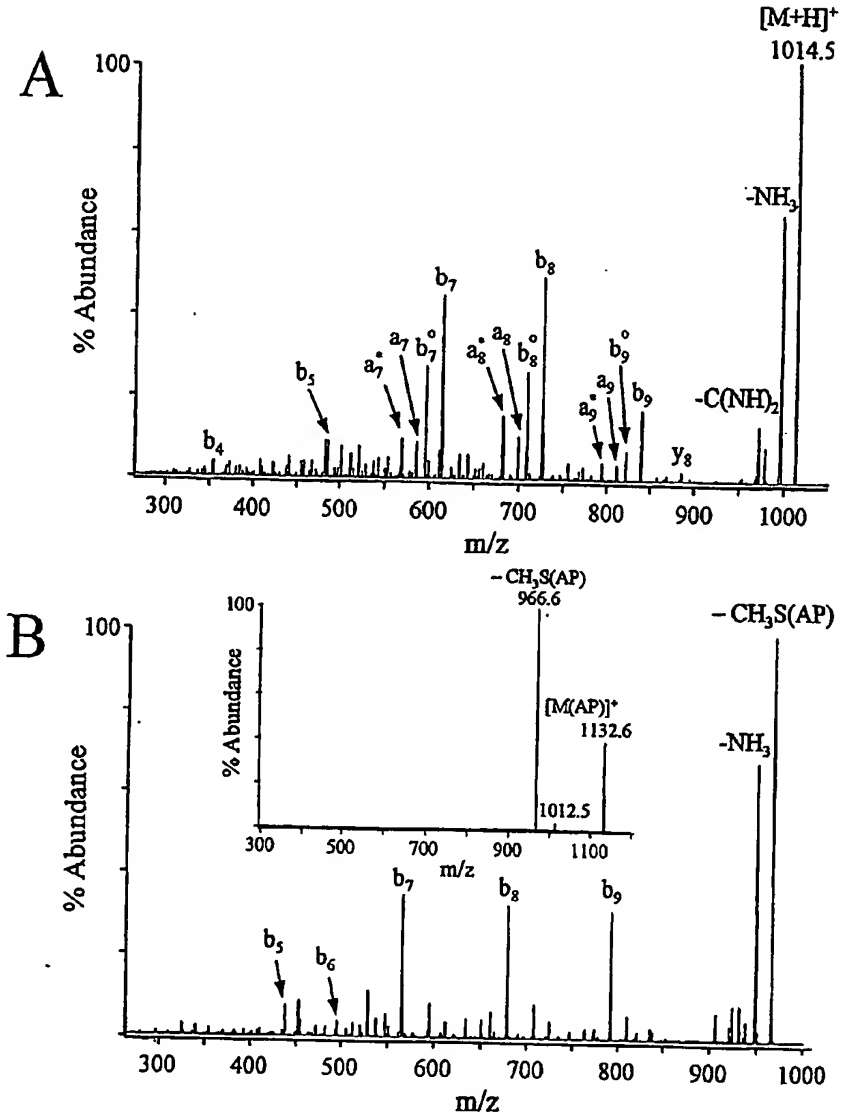

A
100
% Abundance
[M+H]+
1014.5
-NH3
-C(NH2)2
b4
b5
a7*
a7
b7°
b7
a8*
a8
b8°
b8
a9*
a9
b9°
b9
y8
300
400
500
600
700
800
900
1000
m/z
B
100
% Abundance
- CH3S(AP)
966.6
[M(AP)]+
1132.6
1012.5
300
500
700
900
1100
m/z
- CH3S(AP)
-NH3
b5
b6
b7
b8
b9
300
400
500
600
700
800
900
1000
m/z

Figure 6

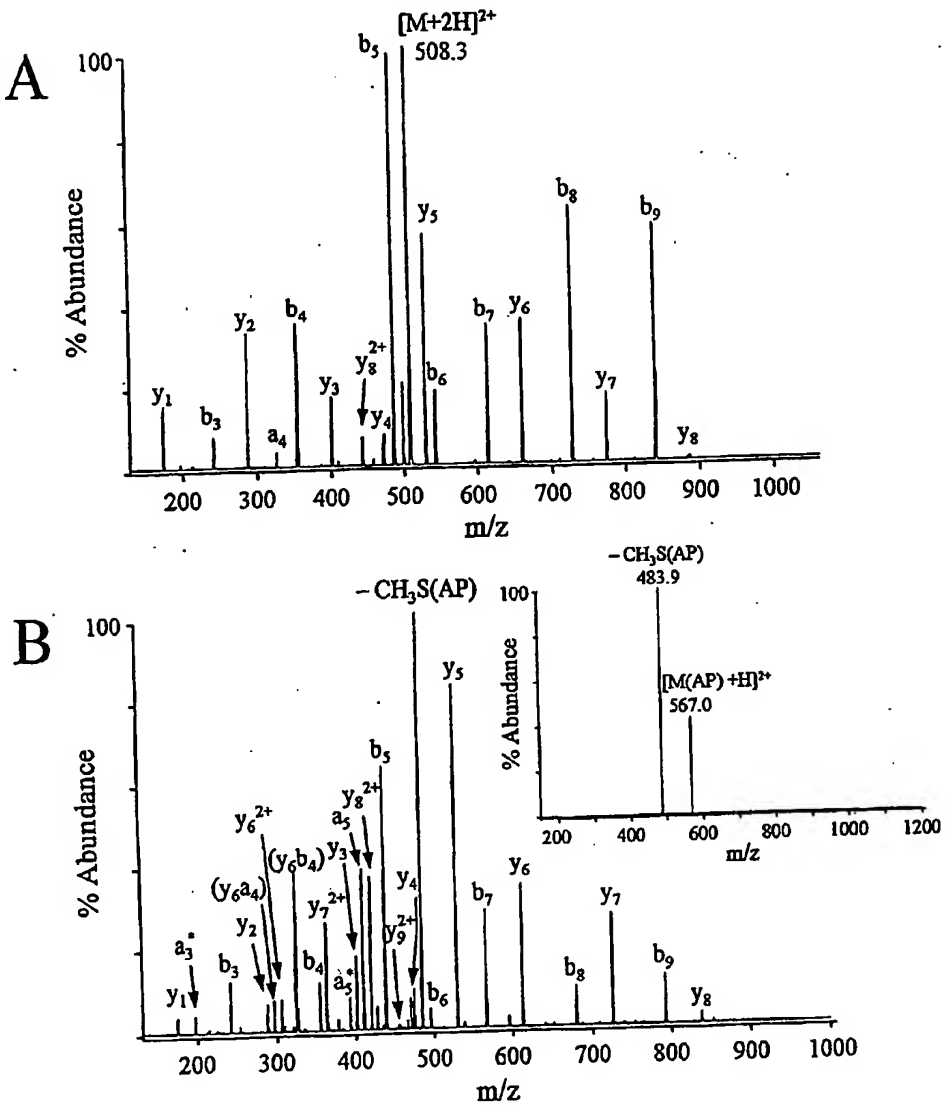

A
[M+2H]2+
508.3
100
% Abundance
b5
y5
b8
b9
y2
b4
b7
y6
y8 2+
b6
y7
y1
b3
y3
y4
a4
y8
200
300
400
500
600
700
800
900
1000
m/z
B
-CH3S(AP)
100
% Abundance
y5
b5
y8 2+
y6 2+
a5
y3
(y6b4)
y4
y6
b7
y7
(y6a4)
y7 2+
y2
y9 2+
a3*
b3
b4
a5*
b8
b9
y1
b6
y8
200
300
400
500
600
700
800
900
1000
m/z
-CH3S(AP)
483.9
100
% Abundance
[M(AP)+H]2+
567.0
200
400
600
800
1000
1200
m/z

Figure 7

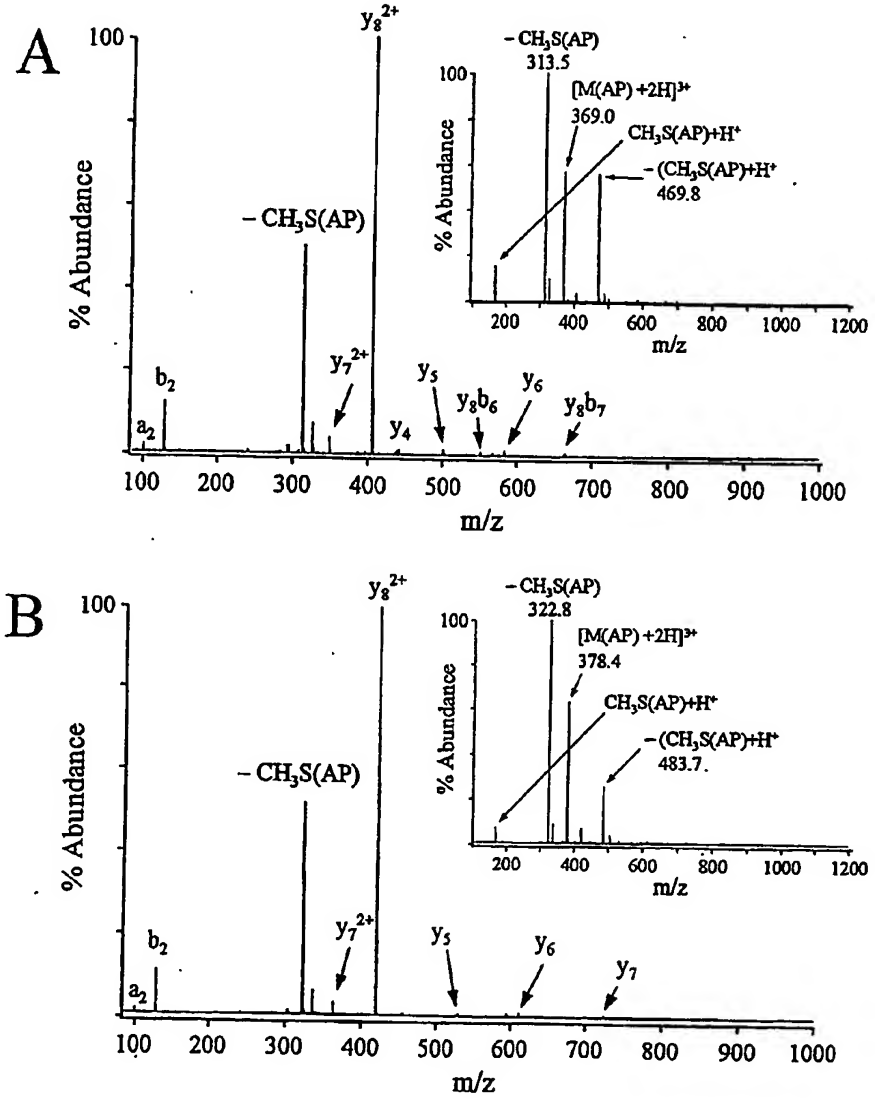

A
100
% Abundance
y_8^{2+}
$-CH_3S(AP)$
b_2
a_2
y_7^{2+}
y_4
y_5
y_8b_6
y_6
y_8b_7
100 200 300 400 500 600 700 800 900 1000
m/z
$-CH_3S(AP)$
313.5
$[M(AP)+2H]^{3+}$
369.0
$CH_3S(AP)+H^+$
$-(CH_3S(AP)+H^+$
469.8
200 400 600 800 1000 1200
m/z
B
100
% Abundance
y_8^{2+}
$-CH_3S(AP)$
b_2
a_2
y_7^{2+}
y_5
y_6
y_7
100 200 300 400 500 600 700 800 900 1000
m/z
$-CH_3S(AP)$
322.8
$[M(AP)+2H]^{3+}$
378.4
$CH_3S(AP)+H^+$
$-(CH_3S(AP)+H^+$
483.7.
200 400 600 800 1000 1200
m/z

Figure 8

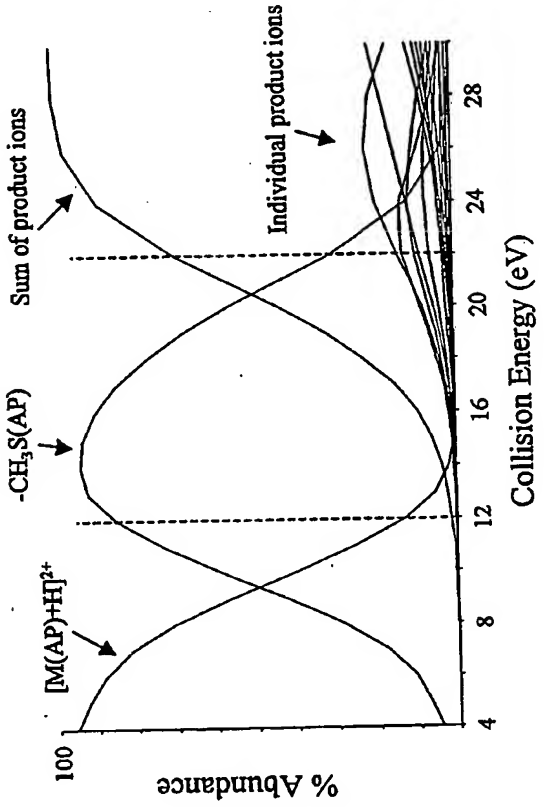

Sum of product ions
Individual product ions
-CH3S(AP)
[M(AP)+H]2+
100
% Abundance
4
8
12
16
20
24
28
Collision Energy (eV)

Figure 9

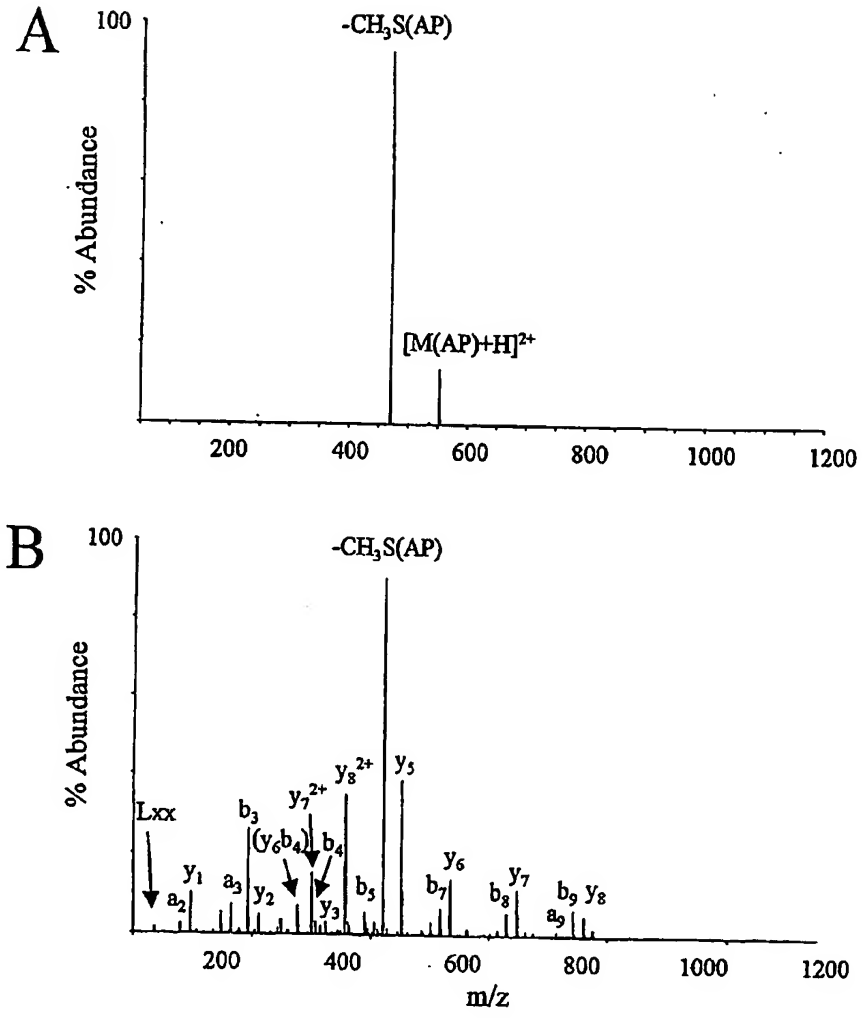

A
100
-CH3S(AP)
% Abundance
[M(AP)+H]2+
200
400
600
800
1000
1200
B
100
-CH3S(AP)
% Abundance
Lxx
b3
y7 2+
y8 2+
y5
(y6b4)
b4
y6
y1
a3
y2
b5
b7
y7
b8
b9 y8
a2
y3
a9
200
400
600
800
1000
1200
m/z

Figure 10

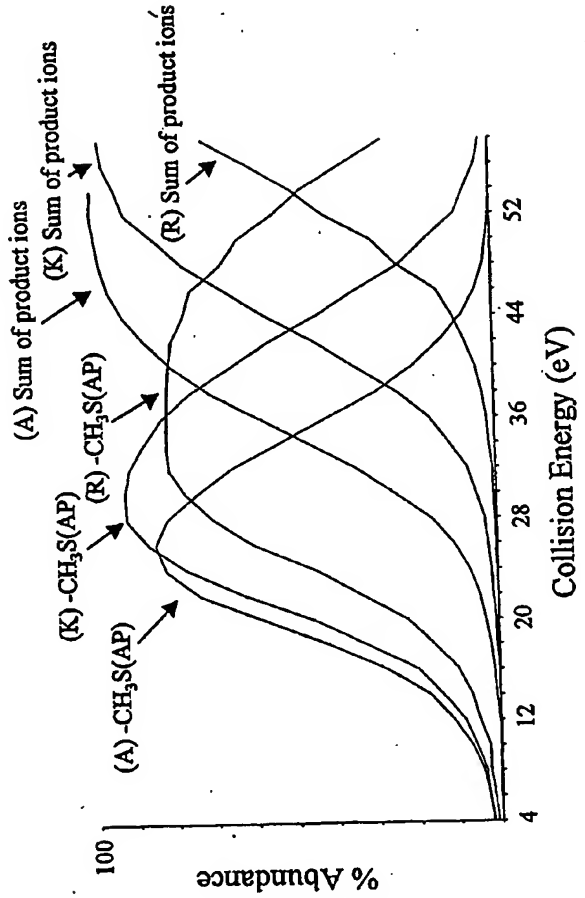

(A) Sum of product ions
(K) Sum of product ions
(R) Sum of product ions
(K) -CH3S(AP)
(R) -CH3S(AP)
(A) -CH3S(AP)
100
% Abundance
4
12
20
28
36
44
52
Collision Energy (eV)

Figure 11

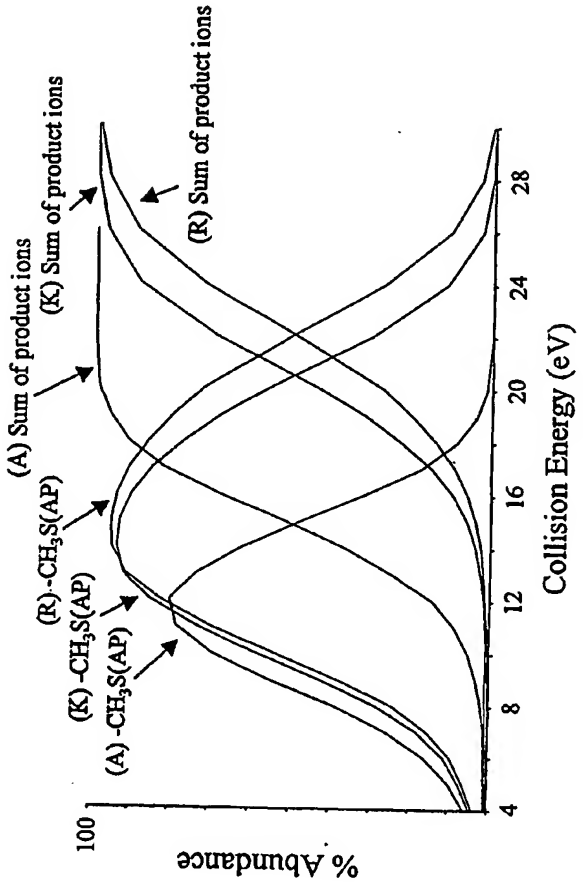

% Abundance
100
4
8
12
16
20
24
28
Collision Energy (eV)
(A) -CH3S(AP)
(K) -CH3S(AP)
(R)-CH3S(AP)
(A) Sum of product ions
(K) Sum of product ions
(R) Sum of product ions

Figure 12

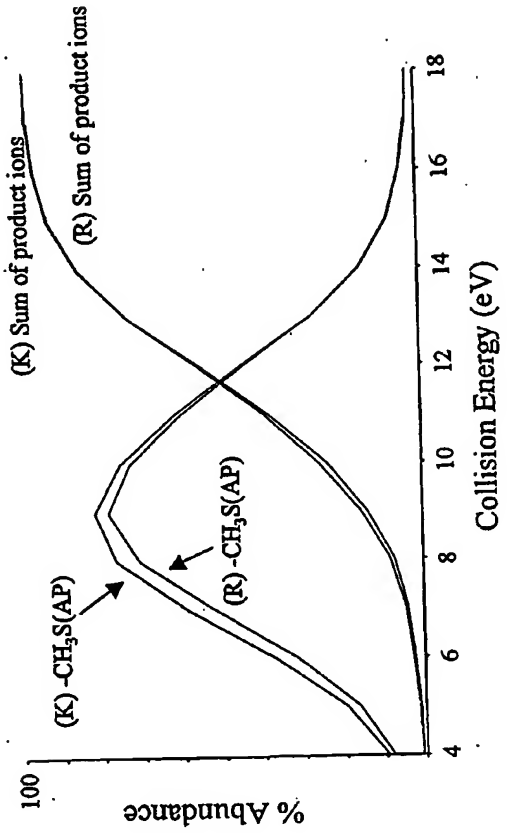

(K) Sum of product ions
(R) Sum of product ions
(K) -CH3S(AP)
(R) -CH3S(AP)
100
% Abundance
4
6
8
10
12
14
16
18
Collision Energy (eV)

Figure 13

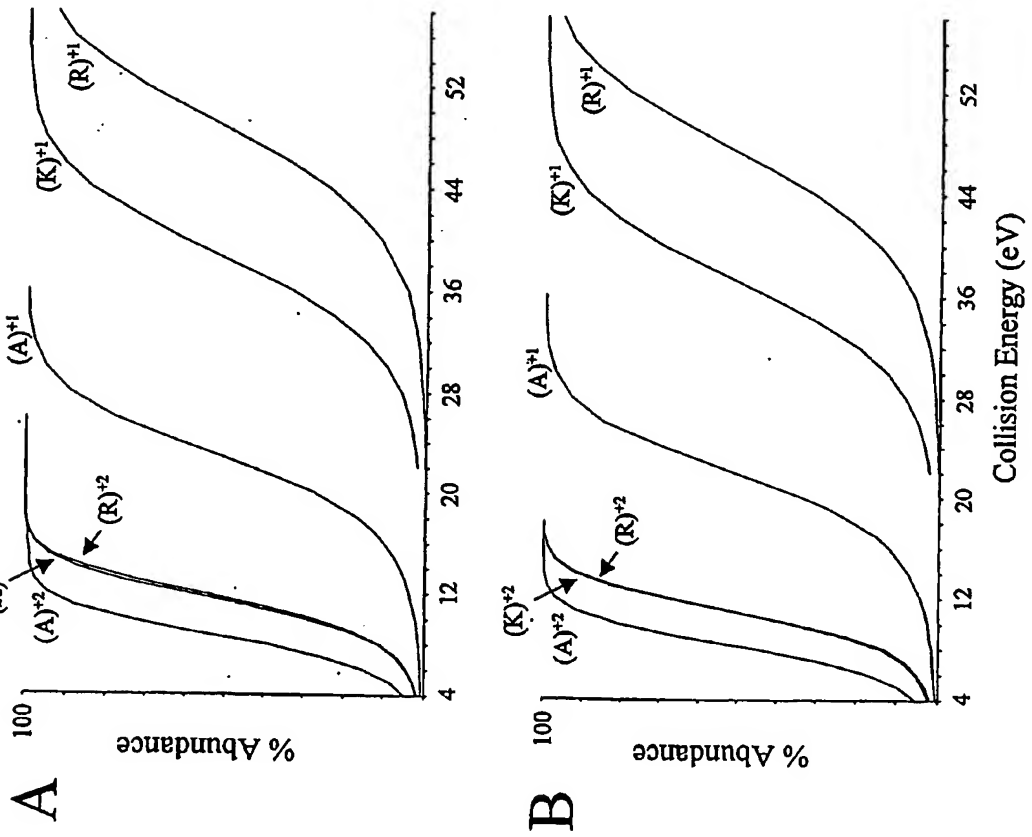

A
B
% Abundance
100
4
12
20
28
36
44
52
Collision Energy (eV)
(K)+2
(A)+2
(R)+2
(A)+1
(K)+1
(R)+1

Figure 14

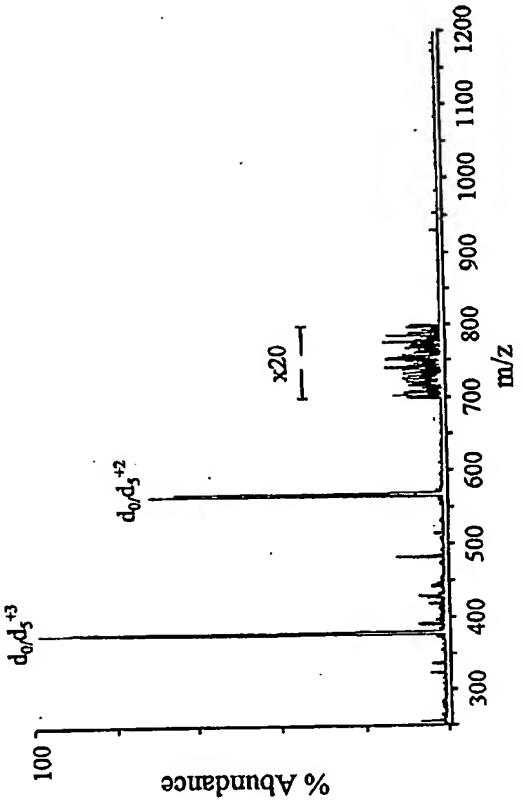

% Abundance
100
300
400
500
600
700
800
900
1000
1100
1200
m/z
d_0/d_5^{+3}
d_0/d_5^{+2}
x20

Figure 15

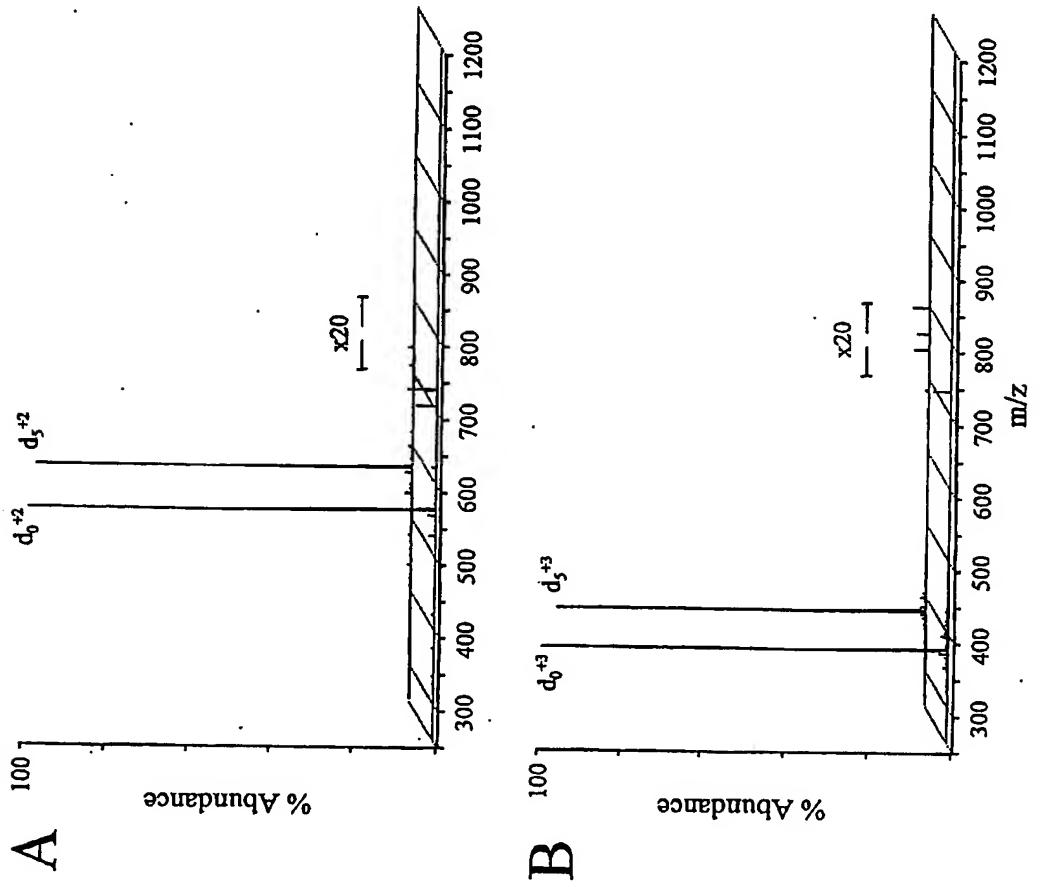

A
% Abundance
100
d_0^{+2}
d_5^{+2}
x20
300
400
500
600
700
800
900
1000
1100
1200
B
% Abundance
100
d_0^{+3}
d_5^{+3}
x20
300
400
500
600
700
800
900
1000
1100
1200
m/z

Figure 16

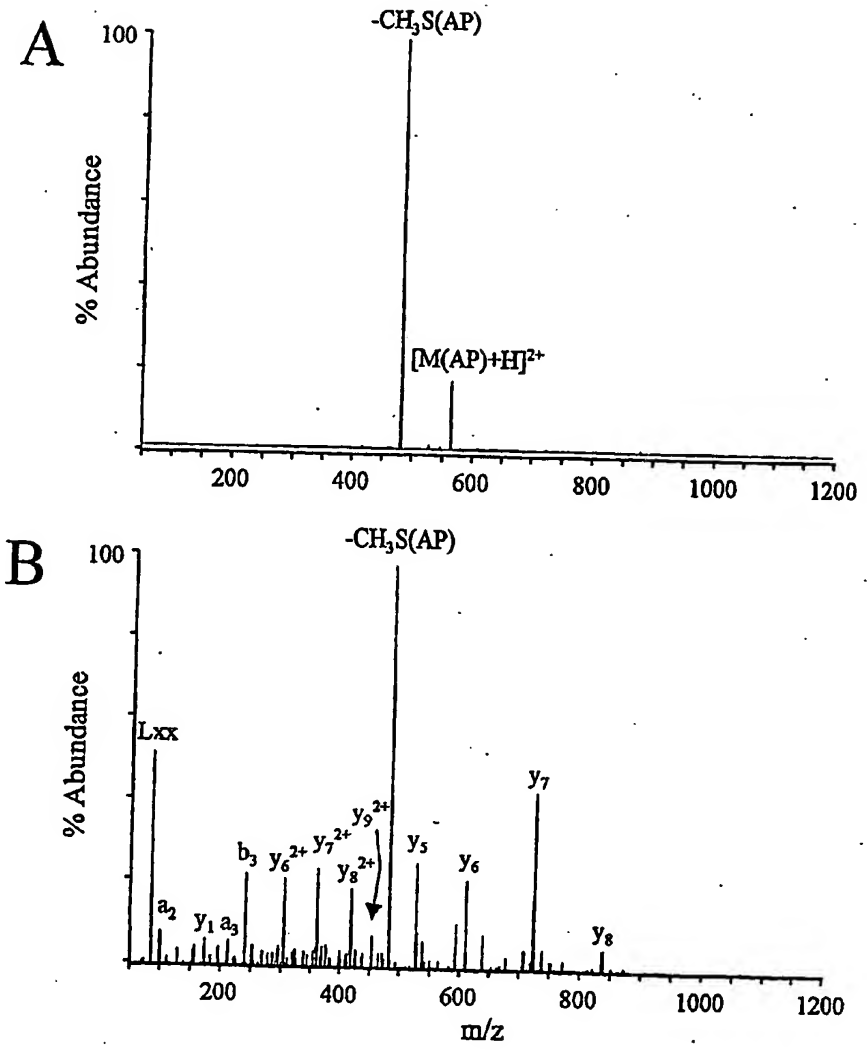

A
100
-CH3S(AP)
% Abundance
[M(AP)+H]2+
200
400
600
800
1000
1200
B
100
-CH3S(AP)
% Abundance
Lxx
y7
y92+
b3
y62+
y72+
y82+
y5
y6
a2
y1
a3
y8
200
400
600
800
1000
1200
m/z

Figure 17

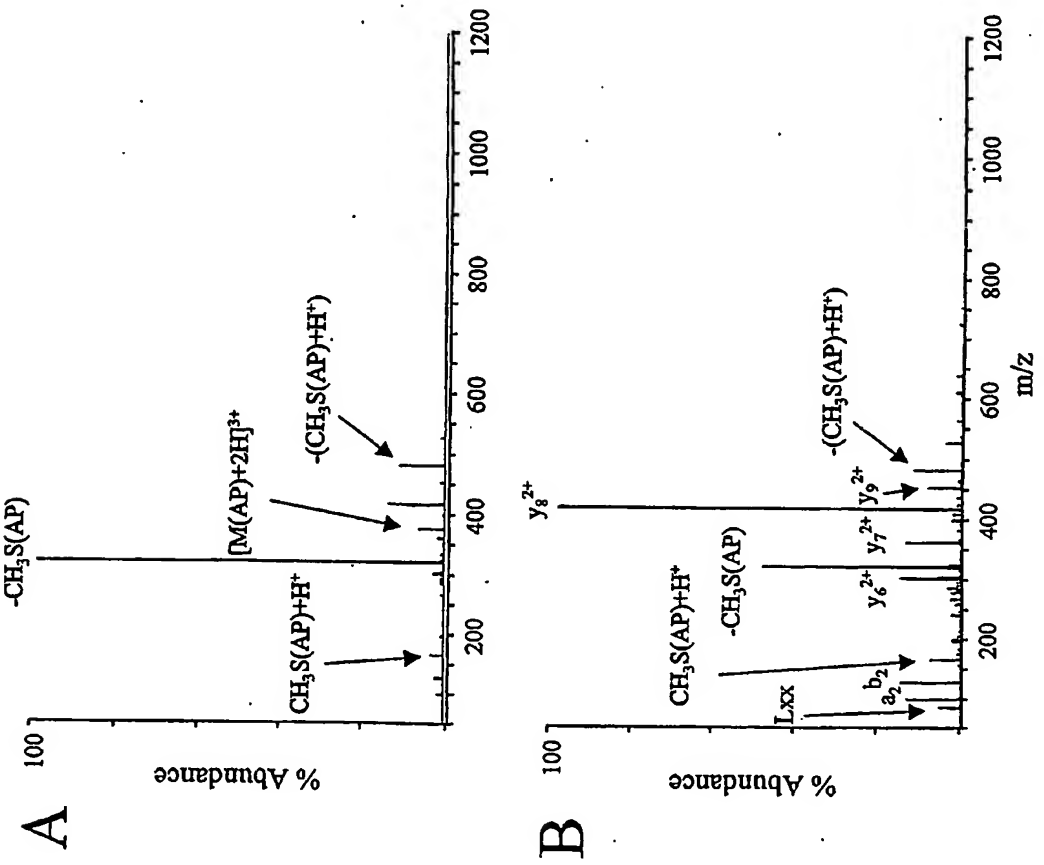

A
% Abundance
100
200
400
600
800
1000
1200
-CH3S(AP)
[M(AP)+2H]3+
-(CH3S(AP)+H+)
CH3S(AP)+H+
B
% Abundance
m/z
y8 2+
CH3S(AP)+H+
-CH3S(AP)
-(CH3S(AP)+H+)
Lxx
b2
a2
y6 2+
y7 2+
y9 2+

Figure 18

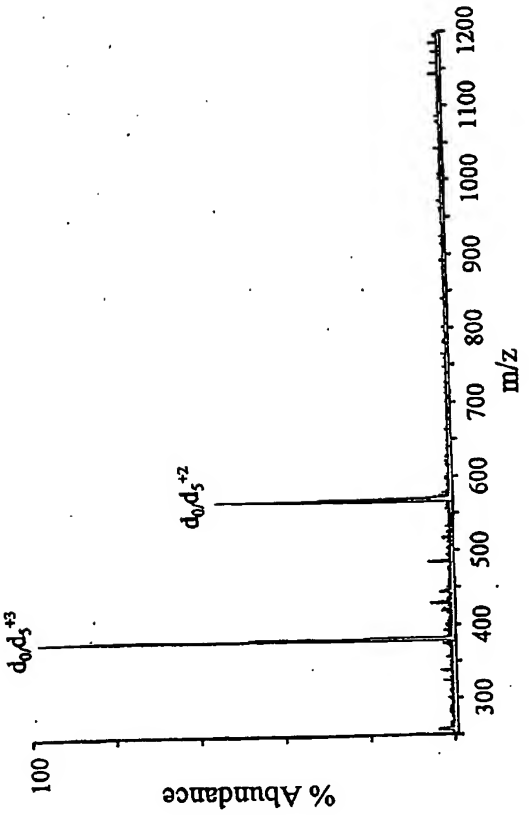

% Abundance
100
300
400
500
600
700
800
900
1000
1100
1200
m/z
$d_0d_5^{+3}$
$d_0d_5^{+2}$

Figure 19

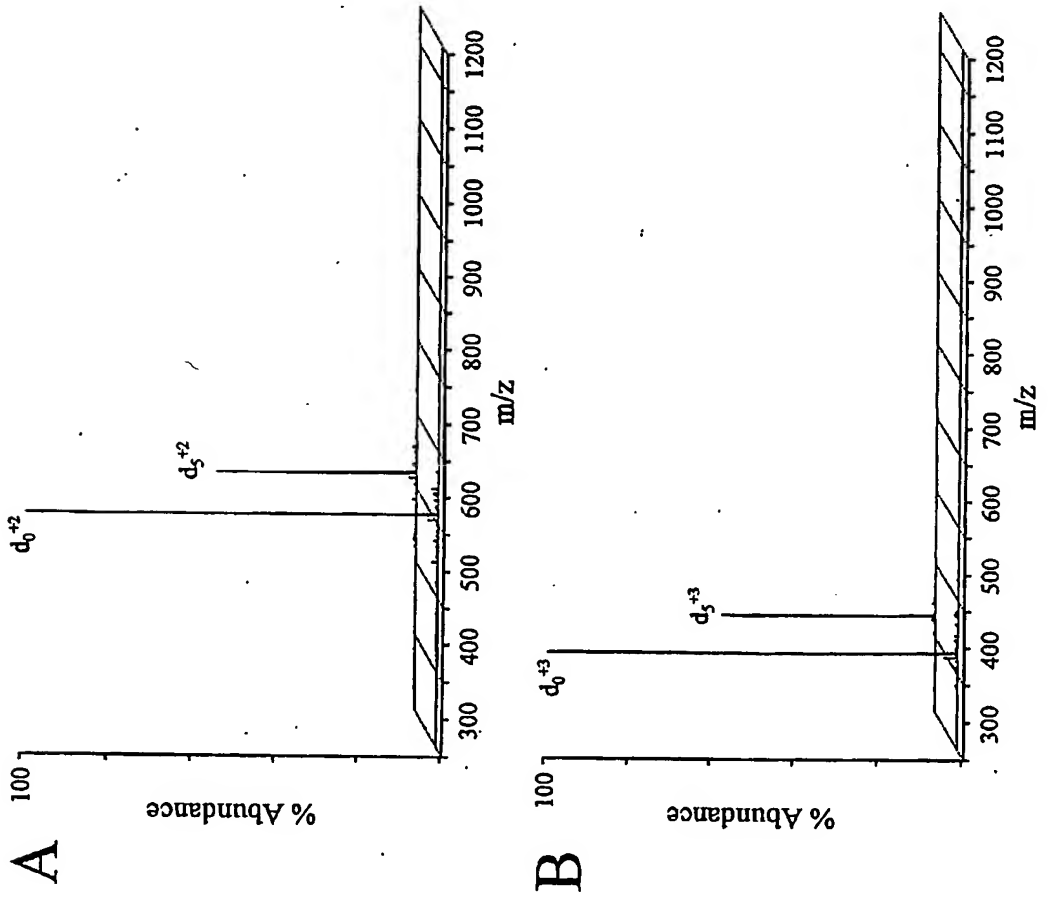

A
% Abundance
100
300
400
500
600
700
800
900
1000
1100
1200
m/z
d_0^{+2}
d_5^{+2}
B
% Abundance
100
300
400
500
600
700
800
900
1000
1100
1200
m/z
d_0^{+3}
d_5^{+3}

Figure 20

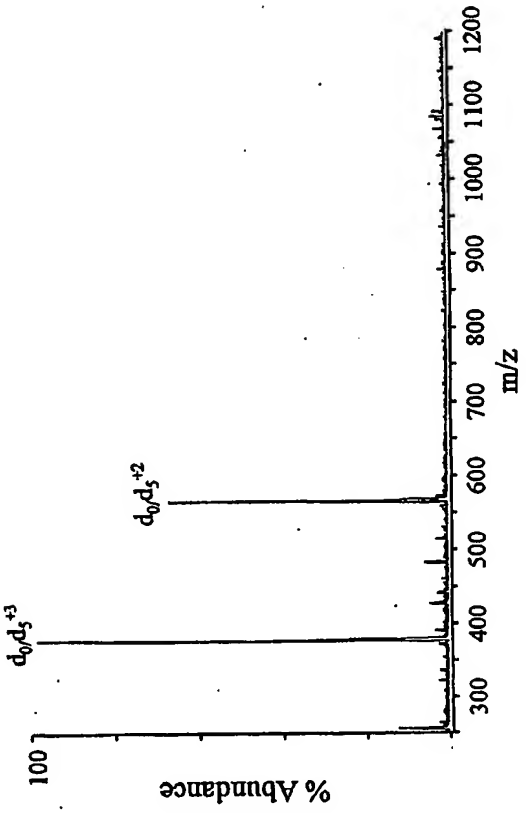
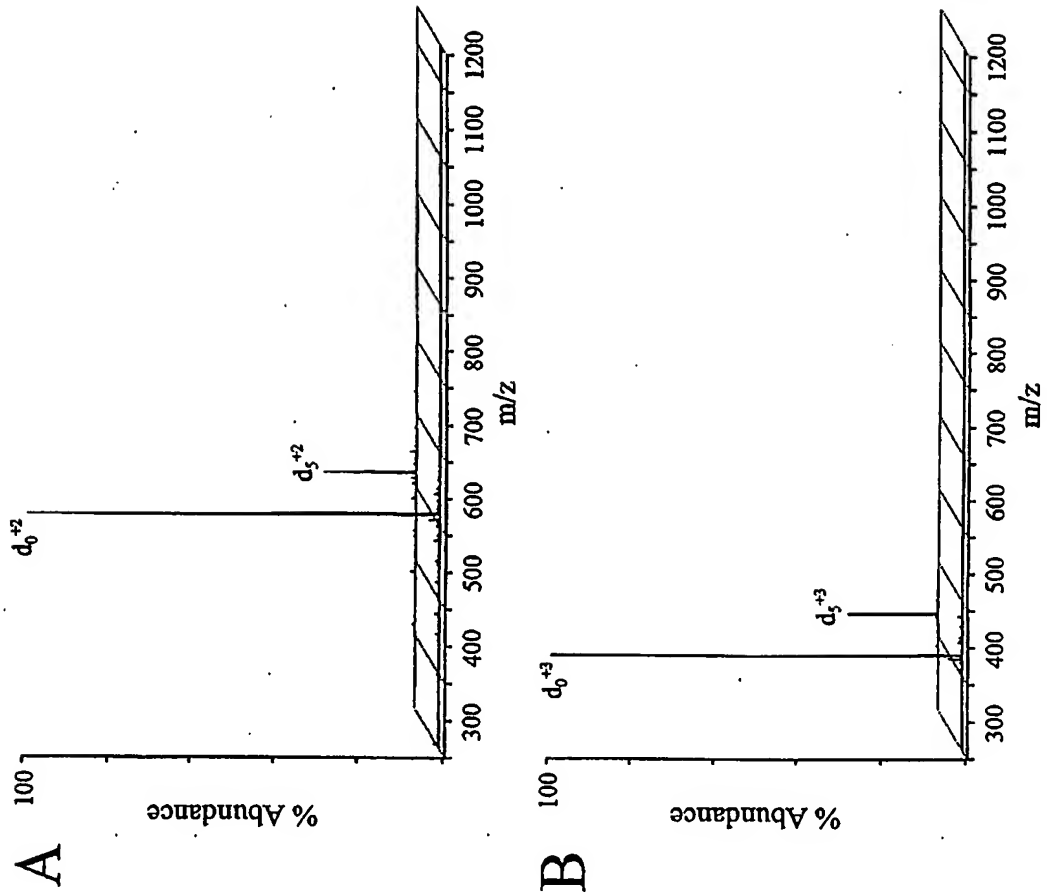

% Abundance
100
$d_0d_5^{+3}$
$d_0d_5^{+2}$
300
400
500
600
700
800
900
1000
1100
1200
m/z

Figure 21


A
% Abundance
100
d_0^{+2}
d_5^{+2}
300
400
500
600
700
800
900
1000
1100
1200
m/z
B
% Abundance
100
d_0^{+3}
d_5^{+3}
300
400
500
600
700
800
900
1000
1100
1200
m/z


PCT Application
US0336739